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The epidemiology of bovine tuberculosis and influence of liver fluke co-infection in Cameroon, Central Africa.

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"Surely it is a braver, a saner and a truer thing to rebel than to tamely accept it as the natural lot of men."

Sir Roger Casement CMG

Anglo-Irish Humanitarian 1864-1916.

The road back and forth to Nwa.

(Donga Mantung, North West Region, Cameroon. March 2013.)

Declaration

This thesis is submitted to the University of Edinburgh in accordance with the requirements for the degree of Doctor of Philosophy in the Faculty of Medicine and Veterinary Medicine. The project was funded by the Wellcome Trust and part-time PhD fees were self-funded. All work in this thesis is entirely my own original work unless otherwise stated. The work in this thesis that was not conducted by myself includes:

1. **Chapter 3** (Abattoir and cross-sectional studies): Original concept of a larger Wellcome Trust funded project and study design by B. M. de C. Bronsvoort, V. Tanya and K. L. Morgan.
2. **Chapter 3** (Abattoir study): Collection of samples in Ngaoundere abattoir was conducted by N. F. Egbe, S. Mazeri, A. Muwonge, A. Novak and K. L. Morgan.
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4. **Chapter 5** (Abattoir study): Bayesian estimation of IFN-γ assay sensitivity was conducted by B. M. de C. Bronsvoort and I.G. Handel.

Signed:

Robert Francis Kelly

Thesis lay summary

Bovine tuberculosis (bTB) is an infectious disease of cattle caused by *Mycobacterium bovis*. Zoonotic transmission of *M. bovis*, from cattle to people, can potentially lead to debilitating disease and is the main driver for disease control in cattle. People contract *M. bovis*, by living in close contact with cattle or by drinking raw milk. Thus primarily public health is usually protected through pasteurisation of milk and identifying infected cattle for removal in test and slaughter programs. In high income countries bTB is either eradicated or controlled to a low level through test and slaughter programs, with are reliant upon accurate diagnosis of infected cattle. Diagnosis of infection in live cattle is difficult because clinical signs, such as pneumonia and weight loss, do not always develop, are not very specific and commonly animals can appear healthy. Progression of disease leads to characteristic abscesses developing in organs of infected cattle, such as the lungs and lymph nodes, however they can only be identified post mortem. Subsequently bTB diagnosis is dependent upon detecting immune responses to *M. bovis* in live cattle. The predominant response is the cell-mediated immune response and can be detected using the single comparative intradermal skin test (SCITT) or the interferon-gamma (IFN-γ) assay. The IFN-γ assay is potentially useful for epidemiological studies, as it only requires a single blood sample and cattle can be tested on mass. However neither test is 100% accurate, with accuracy varying between cattle populations, and false negative results are common especially in early or late stage infections. It is not completely known why false negative IFN-γ assay test results occur and false negative results can ultimately lead to underestimation of bTB prevalence. Liver fluke parasites may dampen down IFN-γ immune responses to *M. bovis* resulting in bTB false negative test results in co-infected cattle, although this has been minimally investigated in natural infection settings.

Cattle production is integral to many rural livelihoods in low-middle income countries. Yet bTB can be a serious public health risk in pastoral and small holder cattle-rearing systems. For example in the Central African country of Cameroon, keeping cattle is economically important to pastoral communities and in the past 10 years a dairy industry has continued to grow with increased demands for both meat and fresh milk. Although bTB is present in Cameroonian cattle the magnitude of the problem is unclear. The IFN-γ assay may be useful in detecting infected cattle to describe the epidemiology of bTB in Cameroon. However diagnostic test performance needs to be investigated prior to estimating the proportion of bTB positive cattle and why they became infected.

Firstly the accuracy of the IFN-γ assay was tested in Cameroonian cattle by sampling 2064 cattle in two slaughter houses based in the cities of Bamenda (North West Region; NWR) and Ngaoundere (Vina Divison; VD) in 2012-13. The results of the IFN-γ assay, with a selected cut-off value of $\geq$0.1, were compared to slaughtered cattle post mortem results and other blood test results to try to explain false negative results. Comparison showed that differences in liver fluke infection may account for the test sometimes missing *M. bovis* infection. Post mortem results also showed that liver fluke infection may increase the development of bTB pathology and underestimate the proportion detected by the IFN-γ assay by $\sim$20%. To see if liver fluke affected the accuracy of diagnosing bTB in the field, using the IFN-γ assay, a liver fluke blood test was developed for use in live cattle.

Secondly a survey of live cattle in Cameroon was conducted to highlight the public health risk of bTB, estimate the proportion of cattle infected with bTB considering liver fluke co-infection and identify why cattle might be infected. In total 1498 cattle were sampled from 100 pastoral herds, in the NWR and VD, which have been traditionally kept in Cameroon for centuries. Cameroon has a small but growing dairy

industry and the cattle breeds and management are completely different to the pastoral system, therefore, a separate sample of 60 cattle were screened from 46 small scale dairy farmers in the NWR. All cattle were tested for bTB using the IFN-γ assay, SCITT for comparison and information was collected about how cattle were reared. Awareness that *M. bovis* could be transmitted to people from cattle was low yet drinking of raw milk was commonplace, highlighting the potential food hygiene risk of *M. bovis* transmission to cattle keepers. It was found that the proportion of dairy cattle with bTB was higher (21.67%) than pastoral cattle in the NWR (11.33%) or VD (6.55%) based on the IFN-γ assay. But the proportion of pastoral cattle with bTB could be higher as liver fluke infection was common. Size of the herd and cattle management practices were identified as factors which might influence cattle being positive for bTB in the two systems.

Looking to the future, evidence presented in this thesis could be used as a platform for improvement of bTB control policies in cattle and human populations in Cameroon. Furthermore the impact of liver fluke co-infections on bTB diagnosis may hinder control of bTB where the parasite is present, in cattle populations globally.

Thesis abstract

Despite Africa accounting for ∼20% of the global cattle population, prevalence estimates and related risk factors of bovine tuberculosis (bTB), caused by *Mycobacterium bovis*, are still poorly quantified in many countries across the continent. Control of bTB in Africa is difficult due to poor monitoring of cattle movements and limited abattoir surveillance. Also *M. bovis* is zoonotic and risk factors for transmission include living in close contact with cattle and consumption of unpasteurised milk. Cattle keeping is integral to some rural populations in Cameroon and understanding the epidemiology of bTB in cattle populations is important both to bovine and public health. Detection of bTB in cattle is difficult due to variability of immune responses to *M. bovis* infection. The interferon-γ (IFN-γ) assay maybe useful to estimate bTB prevalence and identify bTB risk factors in Cameroon. However its performance can vary at different stages of bTB pathogenesis and in different cattle populations. Recently *Fasciola hepatica* co-infections have been reported to suppress IFN-γ responses in *M. bovis* infected cattle but the potential effect with *F. gigantica* co-infections on bTB prevalence estimates in Cameroon is unknown.

An abattoir study was conducted in Cameroon to assess the performance of the IFN-γ assay. In 2012-13; 2064 slaughtered cattle were sampled from Bamenda abattoir (North West Region; NWR) and Ngaoundere abattoir (Vina Division; VD). Individual animal data was collected from routine meat inspection including identification of bTB and *Fasciola* pathology. Cattle were also tested for bTB using the IFN-γ assay and an *M. bovis* antibody ELISA. In the absence of a gold-standard diagnostic, the IFN-γ assay was compared to other diagnostic tests to assess agreement and identify factors that affected performance of the assay. Agreement between IFN-γ assay, TB lesion identification and an *M. bovis* antibody ELISA was poor-moderate, probably partly related to differences in immune response detected. A presence of *Fasciola*

gigantica also increased the odds of false negative IFN-γ assay results. On further investigation co-infected cattle had increased odds of TB lesions and reduced IFN-γ responses that potentially could lead to $\sim$20% reduction in test sensitivity. In an attempt to take into account the potential impact of *F. gigantica*, when estimating bTB prevalence, an antibody ELISA was developed to detect the exposure in live cattle.

To highlight the awareness of disease in cattle-rearing communities, estimate prevalence and identify risk factors of bTB in cattle populations; two cross-sectional studies were conducted in 2013. A stratified clustered cross-sectional study of pastoral cattle herds, in the NWR and the VD, sampled 1448 pastoral cattle reared by 100 pastoralists. A smaller cross-sectional study sampled 60 dairy cattle from 46 small-holder co-operative dairy farmers. Individual animal data and herd-level data were collected and animals were screened by both the single comparative intradermal skin test (SCITT) and IFN-γ assay. Awareness of zoonotic TB was low yet consumption of raw milk was high in cattle-keeping communities highlighting the need for accurate bTB prevalence estimates. Despite the high awareness of the clinical presentation of bTB, clinical signs identified by pastoral herdsmen were not associated with cattle being bTB positive. The SCITT was used to compare two manufacturers cut offs for the IFN-γ assay, $\geq$0.05 and $\geq$0.1, and highlighted that these two diagnostics may detect different populations of bTB positive cattle. Using the IFN-γ assay at $\geq$0.1, bTB prevalence was highest in dairy cattle (21.67%) and was also present in pastoral cattle in the NWR and VD (11.33% and 6.55% respectively). Importantly, as *F. gigantica* is endemic in Cameroon and its influence could mean the true prevalence of bTB could be higher. Female pastoral cattle were at lower odds of being IFN-γ assay positive potentially due to immunosuppressive factors had lower odds of disease. Husbandry practices also decreased the odds of being IFN-γ assay positive such as drinking from streams, antelope and contact with

herds at grazing. Age increased the odds of pastoral cattle being IFN-γ assay positive potentially being a confounder to chronicity of bTB and other co-infections may influence IFN-γ responses. Dairy cattle herds had different risk factors for being IFN-γ positive likely due to differences in husbandry practices.

Considering the potential risk to public health of *M. bovis* this thesis highlights the extent of bTB across two major cattle keeping regions in Cameroon and the public health risk in cattle-rearing communities. Furthermore the relationship between *Fasciola* co-infection and IFN-γ responses to *M. bovis* described has potential implications for bTB diagnosis in cattle populations where the parasite is present across the globe.

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Abbreviations

AFB: Acid fast bacilli

AI: Artificial insemination

AIC: Akaike information criterion

ANOVA: Analysis of variance

ADCC: Antibody-dependent-cell-cytotoxicity

AUC: Area under the curve

BCG: Bacille calmette-guerin

BCS: Body condition score

bTB: Bovine tuberculosis

CAR: Central African Republic

CBPP: Contagious bovine pleural pneumonia

cfu: Colony-forming unit

CI: 95% Confidence interval

DC: Dentition score

DNA: Deoxyribonucleic acid

DR: Direct repeats

ELISA: Enzyme-linked immunosorbent assay

ESP: Excretory/ secretory proteins

FWEC: Faecal worm egg count

FMD: Foot and mouth disease

GIT: Gastro-intestinal tract

GST: Glutathione S-transferase

HDI: Highest density interval

IG-: Immunoglobulin-

IRAD: Institute of Agricultural Research for Development

IFN-γ: Interferon gamma

IL-: Interleukin-

ITT: Indonesian thin-tail

κ: Cohens kappa statistic

LEID: Laboratory of Emerging Infectious Diseases, University of Buea, Buea, Cameroon.

LJ: Lowenstein-Jensen

LN: Lymph node

mamsl: Meters above mean sea level

MCA: Multiple correspondence analysis

MDR: Multi-drug resistant

MGIT: Mycobacteria Growth Indicator Tube

MINEPIA: Ministry of Livestock, Fisheries and Industrial Agriculture

MINREST: Ministry of Scientific and Technical Research

MIRU-VNTR: Multilocus Variable Number Tandem Repeat

MLR: Multivariable logistic regression

MLST: Multilocus sequence typing

MTC: Mycobacteria Tuberculosis Complex

NK: Natural killer

NTM: Non-Tuberculous Mycobacteria

NWR: North West Region

OIE: World Organisation for Animal Health

PBMC: Peripheral blood mononuclear cell

PBS: Phosphate buffered saline

PCA: Principle component analysis

PFGE: Pulsed-field gel electrophoresis

PP: Percent positive

PPD: Purified protein derivative

RAPD: Random amplified polymorphic deoxyribonucleic acid

REA: Restriction endonuclease analysis

RFLP: Restriction fragment length polymorphism

ROC: Receiver operating characteristic curve

R(D)SVS: Royal (Dick) School of Veterinary Studies, University of Edinburgh, Edinburgh, UK.

S/P: Sample to positive ratio

SCITT: Single comparative intradermal tuberculin test

SE: Sensitivity

SIT: Single intradermal tuberculin test

SNP: Single nucleotide polymorphism

SP: Specificity

TMB: 3,3',5,5'-Tetramethylbenzidine chromogenic substrate

TB: Tuberculosis

TBRL: Tuberculosis reference laboratory Bamenda, Bamenda, Cameroon.

TGF-B: Transforming growth factor B

UV: Ultraviolet light

VC: Veterinary Centre or Centre Veterinaire et Zootechnique

VD: Vina Division

WHO: World Health Organisation

ZN: Ziehl-Neeson

zTB: Zoonotic tuberculosis

Chapter 1

Introduction and literature review

1.1 Thesis motivation and objectives

Bovine Tuberculosis (bTB), caused by *Mycobacterium bovis*, is a chronic disease of cattle that can result in poor health and welfare of affected cattle. Subclinical disease is the most common presentation in endemic settings, which is difficult to detect, only presenting as loss of production. *Mycobacterium bovis* infections can be latent with the potential to develop clinical disease over months to years later. Importantly cattle can transmit *M. bovis* to humans. *Mycobacterium bovis* infections is responsible for 3.1% of human TB cases globally, often linked with HIV infections, but the percentage of cases in sub-Saharan Africa (SSA) is unknown. Zoonotic transmission occurs through consumption of raw milk and living in close contact with cattle. Hence communities in SSA that have high contact with cattle and their products, where bTB is present, are at high risk. International legislation is in place to prohibit trade between officially disease free and bTB endemic countries, leading to additional economic impact. The epidemiology of bTB is unknown in cattle populations of many SSA countries and consequently few of these countries have local or national control programs in place.

In Cameroon, Central SSA, cattle are economically and culturally important for many communities. Bovine tuberculosis has been described as endemic and poses a significant public health risk due to high levels of milk consumption across the country. The level of awareness of the disease in cattle and the zoonotic risk is unknown in cattle keeping communities in Cameroon, greater awareness may influence how people consume milk products. Despite the potential zoonotic risk of bTB, meat inspection for TB lesions is the only means of protecting public health, there is no control of bTB in live cattle. Only a few studies have estimated the prevalence of bTB in Cameroon and the wide range of estimates (0.2-40%) is related

to the inadequacy of the diagnostic tests that are currently available.

Ante-mortem diagnostic tests for bTB rely upon detecting immune responses to *M. bovis* infection. These include the intradermal skin tests and the interferon-gamma (IFN-γ)) assay which detect the predominant Th1 response to *M. bovis*. The IFN-γ assay is reported to be more sensitive than the intradermal skin tests and might be useful to investigate bTB epidemiology. However the diagnostic sensitivity and specificity of the IFN-γ assay varies in different settings and the test has not been assessed in Cameroon. Furthermore, experimental studies show co-infection with *Fasciola hepatica* down-regulates the Th1 responses to *M. bovis* infection in cattle. *Fasciola hepatica* co-infection has been shown to decrease the sensitivity of cell-mediated immunity (CMI) diagnostics, such as the IFN-γ assay, and the development of lesions that are important in bTB surveillance and control. *Fasciola* infection is suspected to be endemic in the cattle of Cameroon although the species of *Fasciola* present is unknown. Hence understanding the impact of *Fasciola* co-infections on the ability to detect bTB is important for describing the epidemiology of bTB in Cameroon.

The level of risk of zoonotic transmission of *M. bovis* in cattle rearing communities is unknown. Further understanding of bTB epidemiology in Cameroon is required to protect animal and public health. The main aim of this thesis is to describe the epidemiology of bTB in Cameroonian cattle rearing communities using the IFN-γ assay. In order to achieve this aim, the impact of *Fasciola* co-infections on the IFN-γ assay to diagnose bTB needed to be assessed within Cameroon's cattle population prior describing the epidemiology of bTB.

Chapters 1 and 2 introduce the context and work conducted to achieve the aims of this thesis. Chapter 3 describes the study designs, field and laboratory work conducted.

Specific objectives are explored in each chapter to address the aims of the thesis:

- **Chapter 4**: What are the reasons for false positive and false negative test results when using the IFN-γ assay in Cameroon?
- **Chapter 5**: Does *F. gigantica* co-infection affect diagnosis of bovine tuberculosis in Cameroon?
- **Chapter 6**: Can a newly developed *F. gigantica* ELISA detect *F. gigantica* exposure in cattle?
- **Chapter 7**: What is the current level of awareness of bovine and zoonotic tuberculosis in cattle rearing communities?
- **Chapter 8**: What is the prevalence of bovine tuberculosis in different cattle rearing communities in Cameroon, comparing the IFN-γ assay to SCITT estimates?

The final chapter (Chapter 9) summarises the conclusions drawn from the thesis as a whole and the wider implications of the work.

1.1.1 The CAMbTB Project

This thesis encompasses part of the work from an overarching bTB epidemiological project in Cameroon, funded by the Wellcome Trust, entitled "The Epidemiology and Phylogenetics of Bovine Tuberculosis in Cameroon, Central Africa" (CAMbTB project). The author of this thesis was involved in organising, coordinating and conducting multiple aspects of the larger study in the field in Cameroon but by no means conducted the project alone. The project was a collaborative project involving partners in the UK, Cameroon, Chad and Nigeria. It was planned that three study sites were to be included in the project; 1. the North West Region (NWR), 2. the Vina

Division (VD) in the Adamawa Region and 3.the North and Extreme North Regions of Cameroon. However due to instability and terrorism in 2013 in the Extreme North Region this third study area was not completed. The larger bTB epidemiological project encompassed three types of study:

1. Cattle abattoir studies:
   (a) A convenience based abattoir study of slaughtered cattle in Bamenda (NWR) and Ngaoundere (VD).
   (b) A smaller limited convenience based abattoir study of slaughtered cattle in Garoua (North Region) and Maroua (Extreme North).
2. Cattle cross-sectional studies:
   (a) A population based cross-sectional study of pastoral cattle herds based in the NWR and VD.
   (b) A smaller cross-sectional study of dairy herds in the NWR Region.
3. Human studies:
   (a) A longitudinal study at human TB clinics across the NWR.

In the context of this thesis the abattoir (1.a) and cross-sectional (Pastoral (2.a) and dairy(2.b)) studies were designed to answer specific research questions (Chapter 1). The other aspects of the overarching CAMbTB project, studies 1.b and 3., will not be discussed in this thesis. Additionally the molecular aspects of study 1. will be included in this thesis only where appropriate and referenced to the thesis of N. F. Egbe (University of Calibar, Nigeria) or other published work. Other aspects of the CAMbTB project will be referenced to and relevant parties acknowledged by published papers

1.2 Bovine tuberculosis and *Mycobacterium bovis*

1.2.1 Aetiology

Mycobacteria species belong to the phylum *Actinobacteria* with the genus including over 153 recognised species of bacteria. Most *Mycobacteria* species are aerobic, non-encapsulated, acid-fast and slow growing (1). The genus includes species of major global health importance including human and animal tuberculosis (TB). *Mycobacteria* species can be split in the genus to those that produce tuberculous lesions, *Mycobacterium tuberculosis* Complex (MTC), and non-tuberculous *Mycobacteria* species (NTM) (2). The MTC is a complex of closely related *Mycobacteria* species many of which are pathogenic to animals and man causing tuberculous disease. The NTM group includes *M. avium subspecies paratuberculosis* (the cause of Johne's disease in cattle) and*M. leprae* (the cause human leprosy). Environmental mycobacteria also belong to the NTM group inlcuding *M. avium*, *M. fortuitum* and *M. gordonae* (3). These species can be found infecting humans, animals or contaminating the environment with specific mycobacterial species having a specific host preference (4).

Species of mycobacterium within the MTC, causing tuberculous lesions, include *Mycobacterium bovis*, *M. tuberculosis*, *M. canetti*, *M. microti*, *M. pinepedii*, *M. africanum* and *M. bovis subsp caprae* that are 99.9% similar genetically at nucleotide level (5). Members of the MTC are clonal with evidence of infrequent gene exchange (6) although understanding understanding genetic connections between species lineages enables understanding how mycobacteria spread across the globe (Subsection 1.2.6). In a study involving analysis of *Mycobacteria* speciesl

interspersed repetitive units (MIRU) and Variable Number Tandem Repeats (VNTR); two major clades of MTC were shown to exist. Clades of the MTC are speculated to have emerged 40,000 years ago from the Horn of Africa, expanding geographically with human and animal movements across the globe post domestication of cattle, from one common ancestor (3). Broadly speaking clade 1 containing *M. tuberculosis* and clade 2 containing primary animal pathogens, such as *M. bovis*. *M.tuberculosis* is the primary human pathogen causing human tuberculosis and has occasionally been isolated in other species including cattle (7; 8). *Mycobacterium bovis* is the cause of bovine tuberculosis (bTB), zoonotic tuberculosis (zTB) and has been isolated from numerous other mammalian host species (9; 10; 11). It is proposed that humans initially infected animals with the Mycobacterium ancestral progenitor of these two groups, *M. prototuberculosis*, possibly explaining why *M. bovis* is zoonotic (2). Various *M. bovis* strain types have been identified in different cattle and other species populations (12). Different strains may demonstrate different virulence characteristics and stimulate variable magnitudes of immune response which may vary between populations infected (13; 14). For example clonal complexes, strains of *M. bovis* which are closely related and have very similar spoligotype patterns, have been isolated in specific geographical regions such as Europe (Europe 1), East (African 2) and West Africa (African 1) (9; 10; 11). The clonal complex of strains in West Africa, named African 1 (AF1), have in common the absence of spacer 30 which defines a specific chromosomal deletion in the spoligotyping scheme (10). Due to the clonal nature of *Mycobacteria species* species, identification of individual spacer deletions can be used to define populations through spoligotype (3). AF1 has been identified in Mali, Chad, Nigeria and Cameroon but absent from East Africa and Europe; both of which have their own geographically distinct spoligotype complexes (11; 9).

1.2.2 Significance of *Mycobacterium bovis*

Bovine disease

Mycobacterium bovis infection has been noted in many species, including wild and domesticated animals, but has particular importance in cattle (15; 16). Infection with *M. bovis* does not imply disease, as many animals live with latent infections without clinical signs (17) and the expansive nature of the lesions are ultimately what cause disease. Clinical signs can include inappetence, chronic cough, weight loss and death. Development of clinical signs of disease is dependent upon where bTB lesions caused by *M. bovis* develop. Lesions commonly occur within the pulmonary system and associated lymph nodes (LN) often taking years to develop, hence bTB often follows an unpredictable chronic course. Related production losses are a major concern in cattle such as reduced milk yield, reduced weight gain and abortion (18). Although infection can bring about debilitating disease in cattle the main impetus for control, or eradication, in many countries is the zoonotic potential of *M. bovis*.

Zoonotic disease

Human TB is predominately caused by *Mycobacterium tuberculosis* with 3.3 million cases and 1.5-2 million deaths reported worldwide annually (19; 20). Pulmonary disease is most common with *M. tuberculosis* infections, due to respiratory transmission, are often associated with poor living conditions and immunocompromised individuals (21). Human TB caused by *M. bovis*, referred to as zoonotic tuberculosis (zTB), is estimated to cause approximately 3% of human TB cases (22). Risk of zoonotic transmission is the likelihood of *M. bovis* being transmitted from cattle or food products to humans (23). Although respiratory transmission can occur between cattle and humans, consumption of un-treated milk

has been historically the most common form of transmission (24; 16). Consumption of infected meat and cutaneous transmission from trauma when handling infected carcasses is also possible (25). Human to human transmission is also possible particularly in immunocompromised individuals (19; 26). Zoonotic TB commonly presents as extra-pulmonary disease, associated with milk-borne transmission, and commonly presents as cervical lymphadenopathy or "scrofula" (27). Pulmonary zTB is indistinguishable from TB caused by *M. tuberculosis* (28).

Since the early 20th century, human TB is relatively uncommon in high-income countries with advances in treatment, control and improvements in living conditions (29; 30). In respect to zTB, public health and bovine control measures have caused a rapid decline in high-income countries (31). The situation is very different in low-income countries, such as those in sub-Saharan Africa (SSA), with human TB being the third most important infectious cause of mortality. Sub-Saharan Africa accounts for 16% of TB cases reported worldwide that has been exacerbated by the HIV/AIDS epidemic (32; 20; 33). Specifically in Cameroon 55,000 of TB cases were reported in 2014, in a population of 6,000,000, with 37% linked to HIV co-infections and 15% of notified cases being extra-pulmonary (34). Yet the proportion of pulmonary and extra-pulmonary disease caused by *M. bovis* is currently unknown in Cameroon. In the adjacent country of Nigeria there have been reports of human *M. bovis* infection and in Tanzania 10.8% of patients with cervical adenitis were attributed to *M. bovis*; likely linked to milk consumption (35; 8; 36). Many laboratories in SSA cannot differentiate members of the MTC that may lead to further difficulties in TB diagnosis and treatment especially with the current HIV/AIDS epidemic (37; 38; 39; 40; 25).

Differentiation between *Mycobacterium* species can be important as the mode of transmission and focus of subsequent control is different. Agriculture is the main

form of income in rural sub-Saharan communities with many living in close contact with their livestock and could be at risk of *M. bovis* infection (41; 22). Furthermore increased animal protein consumption, including fresh milk, in many sub-Saharan African countries like Cameroon (42; 43; 44) may encourage zoonotic transmission of *M. bovis*.

Economics and livelihoods

Under the auspice of protecting public health, the World Organisation for Animal Health (OIE) is working towards control and eradication of bTB globally (45). In countries that wish to trade cattle internationally, OIE polices stipulate national governments must be in the process of controlling bTB to trade cattle. In many of these high-income countries prevalence of zTB is low (29). Control and eradication of bTB can be a financial burden to cattle trading countries, partly because bTB control measures require a sustained approach due to chronicity of *M. bovis* infections (46). For example bTB eradication of bTB has been ongoing since the 1940s in the UK and although is controlled, from a public health perspective, *M. bovis* has not been eradicated. Such sustained control measures have cost the UK government £500 million between 2004-2014 alone and are mainly funded through public taxation (47). Hence arguably the focus of eradicating bTB for cattle industry and government within these countries is to facilitate international trade rather than protect public health (48).

In sub-Saharan African countries there are few control measures and policies against bTB (22). Subsequently endemic bTB does not restrict local trade but does limit access to international markets and, along with sub-clinical disease, will impact on economic development of cattle rearing communities (43). Additionally transmission

of *M. bovis* to wildlife in SSA can also have knock on consequences to local ecosystems potentially through loss of biodiversity and economically through loss of tourism (49). Few studies have accounted for the economic impact of bTB in cattle in SSA. A study in Ethiopia tried to estimate the costs of bTB to the country's cattle industry, taking into account loss of production and trade costs, but limited conclusions were drawn due to lack of information (50). Livestock production is not only integral economically to communities but also to individual livelihoods in SSA (51). For example cattle are integral to many pastoralists, such as the Fulani in Cameroon, for wealth, sustenance and culture (52; 53). Due to their close association with cattle there is a very real risk of contracting *M. bovis* (54). Zoonotic disease would not only be detrimental from a health point of view but also for pastoral livelihoods if individuals cannot rear their cattle due to ill health (55).

1.2.3 Transmission

Cattle can become infected with *M. bovis* via various transmission routes. Route of transmission is thought to influence the development of subsequent pathology and concentration of *M. bovis* in milk (56). The primary route is via aerosol transmission. Aerosol transmission of *M. bovis* is greatly facilitated within droplets of respiratory secretions where bacilli are protected from the environment (57). Transmission will be accelerated, especially when kept in confined spaces, when infected cattle develop clinical disease such as coughing. Aerosol contamination of feed has been shown to be a minor route for *M. bovis* transmission even in intensive husbandry systems (58). Faeco-oral transmission, where bacilli pass onto pasture and fomites from infected cattle faeces, is considered a secondary form of transmission in intensive and extensive husbandry systems (56). Milk transmission occurs, to a lesser extent, and is particularly important in transmission to suckling calves (59; 60). Genital, cutaneous

and congenital transmission are thought to be of minor importance in most settings (61).

Survival of *M. bovis* within the environment will affect transmission. Between 10-25° C, *M. bovis* can survive for up to 18 months under the laboratory conditions (62). Although *M. bovis* has been reported to survive up to two years within a faecal pat in a laboratory setting (63), survival within moist soil is limited to 1-2 months (64). Survival of *M. bovis* is thought to be much shorter in the field as the organism can be destroyed by exposure to UV in sunlight, desiccation and low pHs (62; 58). Water source transmission is possible as the organism survives in moister environmental conditions and splashing of water may facilitate transmission through droplets (57). Resilience of *M. bovis* within the environment might be particularly important for transmission in moist tropical climates, such as SSA, where cattle are grazing pasture with access to natural water sources. Yet the infectiveness of surviving *M. bovis* and importance of different transmission routes has been minimally studied in tropical settings.

From field data, mathematical models have predicted shedding occurs from between 11-87 days post-infection (65; 66). This wide variation is likely dependent upon inoculation dose of *M. bovis* and pathological progression of bTB. A single bacillus within an aerosol droplet is thought to be able to establish infection within the host (67; 41). Inoculation doses for aerosol transmission are thought to be below 92cfu in natural settings (66; 41). Larger doses are thought to be required for faeco-oral transmission, of 10mg of bacilli, compared to aerosol transmission (68). In laboratory settings a range of $1x10^3$-5 $x10^5$ cfu are commonly used for inoculation doses via aerosol (69; 70; 71). In such experiments, cattle which receive larger doses tend to develop more extensive bTB pathology, however it is not clear if cattle with bTB pathology are likely to shed bacilli. Some studies report increased shedding with

increased pathology (72), whilst other studies report shedding uncommon due to the presence of walled off lesions (73). Length of time infected cattle continue to transmit *M. bovis* has been reported for up to 38 weeks however, as few longitudinal studies have been conducted experimentally, excretion may persist beyond this time period (46). For example susceptible cattle housed with infected cattle will not always become infected with *M. bovis* (74), which is likely related to the intermittent shedding of bacilli from latent infections. Many *M. bovis* infections may become latent within the host with limited pathological development which is likely to contribute to poor predictability of *M. bovis* infection outcomes in individual cattle. Recrudesce may occur in later life and these animals, although this is unpredictable, and these cattle can go on to potentially shed *M. bovis*. In addition there is some evidence that some cattle may clear infection (17), making it difficult to be able to predict if infected cattle will transmit *M. bovis* in different cattle populations. Ultimately shedding of *M. bovis* within aerosol secretions will affect transmission rates of the organism and is variable between individual animals throughout the course of infection.

1.2.4 Epidemiology in sub-Saharan Africa and Cameroon

Bovine tuberculosis has a global distribution yet the risk factors for transmission vary between different settings. In low-income countries within SSA the prevalence of bTB in cattle herds is estimated between 0.8% and 49%; the wide range is likely due to the design of studies, different diagnostics used and variations in cattle management practices (75; 35; 76; 77; 57). Similar prevalences were noted in many high-income countries, such as the UK in the early 20th century, prior bTB control measures being in place (78; 28). The prevalence of bTB across extensively managed

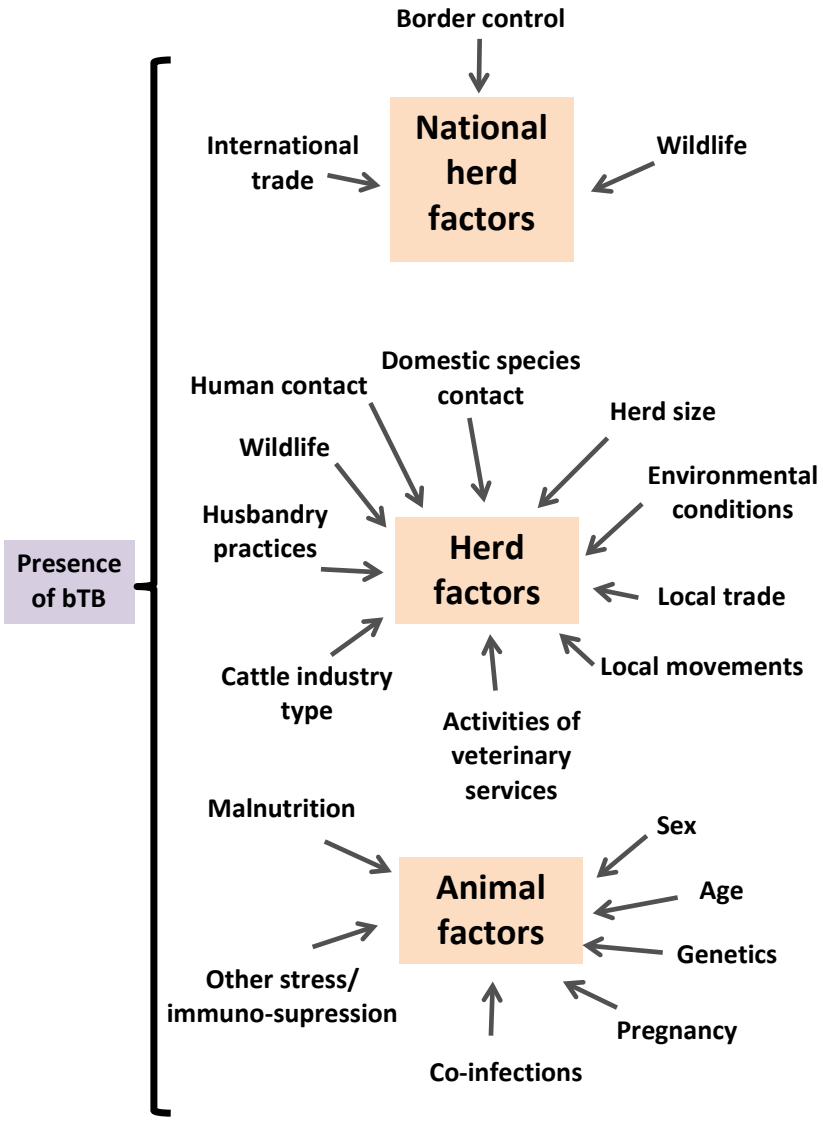


Figure 1.1: **Potential transmission routes of *M. bovis* in sub-Saharan African cattle populations at individual animal, herd and national herd level.**

Schematic diagram complied from the references in this subsection.

pastoral herds in SSA is often high but low within the herd itself (79; 80). Hence within pastoral herds, *M. bovis* transmission is suspected to rely on a few animals maintaining infection within a herd (81). This is converse to the situation in high-income countries, with low herd prevalence and high within herd prevalence (22). Region-specific epidemiological data is limited for bTB for many countries within SSA particularly with few longitudinal estimates (61). Understanding local variation is important as different cattle rearing practices place emphasis on different *M. bovis* transmission routes and risk factors.

It is worth noting that in research studies that investigate bTB epidemiology, the diagnostic tests used to detect *M. bovis* infection or associated immune responses will influence bTB risk factors detected. As different diagnostic tests perform differently through the stages of bTB pathogenesis with none being 100% sensitive or specific. Hence "risk of bTB" is often defined as being test positive to bTB rather than being infectious or presenting with clinical disease. Influence of stage of pathogenesis of bTB and diagnostic test positivity will be discussed in more detail in section 1.2.6.

Environment

Local climate and environment also affect transmission of bTB in SSA whether by respiratory or faeco-oral routes. Seasonal variation has been noted, with wetter seasons where animals are brought together for grazing, housing or security reasons facilitates *M. bovis* transmission when investigated using questionnaire-based-studies (75; 35). Flooding has been noted as a significant bTB risk factor along with sharing of water sources with both domestic and wildlife species. Swampy and humid environments are thought to enable survival of mycobacteria for longer periods

(82; 81). Increased bTB transmission can be associated with contaminated water sources due to faecal contamination of drinking water (41; 35; 83; 79). It is also worth nothing that infections with NTM, present in many cattle rearing environments, may contribute to false positive bTB diagnostic test results (4) (Further discussed in section 1.2.6).

Cattle factors

Female and older cattle are reported to be more likely to develop bTB (82; 84; 80). Increased risk of bTB in female cattle has been associated with reduction in immune responses post-partum caused by increased corticosteroid production (85). Older cattle may have been infected with *M. bovis* for longer and subsequently have longer to develop bTB immune responses and pathology with increasing age, rather than primarily be at increased risk of contracting *M. bovis* (86; 87). In humans, increased prevalence of TB, caused by *M. tuberculosis*, has been noted in the elderly as they are thought to be more likely to develop clinical TB when they become immunosuppressed due to latent infections (88). For example in one study investigating TB prevalence in the population of Arkansas USA, using an intradermal tuberculin test, found a larger proportion of people older than 65 years presented with clinical TB than people less than 65 years (89). Another study highlighted that in the USA 2009-2013, older TB patients (45+ years) had a higher mortality rate to TB than younger patients (90). It is common for cattle to be culled prematurely for meat production (2-5 years depending upon the husbandry system (91)) and this might explain why it is uncommon to see clinical bTB in many production systems. Although studies have shown greater proportion of older cattle testing bTB positive using a variety of diagnostic tests. For example a larger proportion of older cattle (1-3 years of age) tested positive for bTB using the single comparative intradermal skin

test (SCITT) during surveillance of the UK cattle population (86) and cattle greater than 2.5 years old were more likely to be bTB positive at post-mortem in an abattoir study in Uganda (87)). Hence older cattle may also be more susceptible to *M. bovis* infection as $\gamma\delta$ T cells may offer some protection against development of bTB in young calves (92; 93). Although how the bovine immune response to *M. bovis* changes in over years has yet to be studied in cattle (explored further in section 1.4.1).

Breed susceptibility has also been noted in high-income countries and much interest has been focused on breeding bTB genetically resistant cattle (94; 95). Some *Bos taurus* breeds, such as Holsteins, have been noted to have increased susceptibility to bTB compared to *B. indicus* breeds (96; 97). Presence of other infections might influence individual cattle immune responses. For example presence of *Fasciola* with *M. bovis* infections can also influence bTB diagnostic test reactivity of individual cattle (98) that is discussed further in section 1.4.

Increased risk of disease in female, older and *B. taurus* breed of cattle might be cofounded due husbandry related factors. For example female cattle are in general kept longer for breeding and often managed differently than their male counterparts (96). Calves fed pooled milk, from multiple cows, can be infected with *M. bovis* even if only one cow is infected and shedding milk from her udder (99; 100; 79; 80). Increased risk of bTB in exotic breeds may also be additive linked with husbandry practices in SSA with high production exotic cattle require more intensive husbandry in confined conditions, favouring respiratory transmission of bTB rather than increased susceptibility (101; 79; 57; 102).

Husbandry systems and trade

A wide range of cattle husbandry systems exist in SSA. They range from extensive pastoral husbandry in areas where there is plentiful pasture to small holder systems in more peri-urban environments (38; 56). Cattle that are herded together into kraals for the night, markets or vaccination programs may facilitate respiratory transmission, through aerosol droplets, due to their close contact (79). Tuberculosis lesions noted in the respiratory tract of cattle at post-mortem examination (PME) provides evidence for such transmission and is known to be the main route of transmission in close-contact intensive systems (61). In semi-intensive peri-urban systems there appears to be an increase in bTB transmission when compared with extensive systems in Ethiopia (80). Specially as high transmission rates have been noted with high stocking densities that commonly occur with intensive and semi-intensive systems (57).

Cattle brought in from outside the herd can increase the risk of *M. bovis* infection. Often cattle are brought in without being tested so their *M. bovis* infection status is unknown. Their origin can be from markets for replacements with local zebu breeds or for genetic improvement of a herd with exotic breeds. Cattle rearing in SSA entails movement of animals within and between countries. For example production systems in SSA may involve nomadic movement, or transhumance, of cattle for pasture provision and for trade of cattle between urban centres (56). It is thought this movement and mixing of cattle will aid transmission of bTB in African communities. The size of herds appears to have variable affect on risk of bTB transmission probably confounded by other associated risk factor, such as interaction with other herds during transhumance, being kralled/ fenced in together, sharing water sources and being under increased stress due to travelling long distances (103; 79). Additionally poor availability of veterinary services in low-income settings, as in much of sub-Saharan

Africa, might influence bTB prevalence due to herdsmen having limited access to disease control knowledge or advise. Although it is worth noting that impact of specific husbandry practices is likely to vary between different cattle populations.

Contact with other species

Transmission of *M. bovis* can occur between cattle, other domestic species, wildlife as well as man (16). In sub-Saharan ecosystems transmission can occur between multiple species where they are in close contact (104). Hence it is commonly reported that where bTB has been eradicated this would not have been possible unless the disease was controlled in other domestic species and wildlife populations (105; 106). Not all species are equally susceptible to *M. bovis*. Maintenance hosts continue to contribute to transmission of *M. bovis* and may influence prevalence within a cattle population. Spillover hosts tend to be infected in the presence of high infection pressures and do not necessarily substantially contribute to *M. bovis* transmission. Some species will play different roles in different ecosystems (107). Often cattle come into close contact with other domestic and wildlife species, many of which are also susceptible to *M. bovis*. Mixing with sheep, goats and camels has been noted to enhance transmission in pastoral herds in Niger and in smallholder homesteads in Tanzania (108; 35). Many wildlife species, especially ruminants and cervids, are suspected to have some role in maintenance of *M. bovis* in pastoral SSA communities where cattle often come into direct contact with wildlife. For example in South Africa wild buffalo (*Syncerus caffer*) populations are routinely tested for bTB with 38% prevalence noted previously (109). As these animals are free-roaming and difficult to restrain for bTB diagnostic testing; controlling *M. bovis* infection in the buffalo population is difficult. Hence buffaloes could act as a potential reservoir for *M. bovis* infection in many cattle populations (57). However in general limited information is

often known about the prevalence of *M. bovis* infection in wildlife populations and dependence upon the composition of wildlife present and species interaction with cattle.

Human to cattle transmission of *M. bovis* is possible where humans are in close contact with cattle (16). It is unclear how frequently this form of transmission occurs in many cattle rearing systems due to difficulties in monitoring human-cattle interactions. Although there are limited studies which highlight transmission in SSA, respiratory routes are likely to be most frequent with humans living in the same air space as cattle (15). Some SSA studies also report *M. tuberculosis* in cattle, postulating transmission from humans in Nigeria and Kenya (8; 110; 111). Although again transmission is largely dependent upon the nature of interaction between cattle and humans, so its importance is likely to vary between settings.

Cameroon

In Cameroon TB was reported in colonial manuscripts, in both human and cattle populations, during the early 20th century yet no impression of prevalence in either population was estimated (112). For example introductions have been postulated, through genotyping studies, to have introduced bTB into West Africa from Cameroon from importation of *Bos taurus* cattle from France during the colonial era from 1917 (113).

Presently the only routine bTB surveillance of bTB in Cameroon is identification of TB lesions at slaughter. Epidemiological studies have estimated TB lesion prevalence from abattoirs in the Littoral, Central, West, North West, North and Extreme North Regions of Cameroon (114; 75; 115; 116). Estimates of TB lesion prevalence in slaughtered cattle range between 0.82-1.3% in the Littoral, West, Central and North

West Regions. Tuberculous lesions in one study were *M. bovis* positive, confirmed by 51% culture and 60% through *M. bovis* serology, highlighting the zoonotic risk to people working with cattle and carcasses (75). Furthermore the prevalence of TB lesions has possibly been increasing since 1995 (0.31%) to 2011 (1.3%) in an abattoir in the Littoral Region in particular (114; 116). A similar increasing trend was noted in the cattle producing North West Region abattoir from 1995 (0.2%) to 2010 (1.6%) (75; 115).

Additionally, genotyping techniques have been used in some studies to investigate the molecular epidemiology of *M. bovis* infections in Cameroon (113; 117; 118). Such genotyping studies, in both cattle and humans, are useful in highlighting the potential transmission routes in both cattle and people. These studies report that epidemiological clusters of genotypes are present in the North West, Adamawa and Northern Regions of Cameroon. Presence of such regional clusters, suggest that *M. bovis* transmission is restricted within each Region potentially related to cattle contacts and movements. For example restriction of cattle movements from 1976 out of the Adamawa province to limit infectious disease transmission into this region might have restricted certain *M. bovis* genotypes to this region. However in reality this restriction appears to have been poorly implemented and has been abolished since the early 2000s (117) and restriction of genotypes might be more related to cattle grazing or trading practices within the Region. Genotyping of human TB cases has also highlighted the presence of zoonotic transmission of *M. bovis* in Cameroon, with a tuberculosis case being identified as *M. bovis* in the West Region (119). Another study identifies *M. tuberculosis* in cattle highlighting the possibility of reverse zoonotic transmission possibly due to close association with humans (118). However all the genotyping studies conducted were based upon convenience samples and are of relatively small sample size. Hence such studies could be unrepresentative of the

cattle or human population at risk of *M. bovis* infection and there is a need for studies with sample representatively from cattle and human populations.

Estimates of bTB prevalence in different Regions of Cameroon are poorly defined and difficult to compare. Despite logistical difficulties attempts have been made to estimate bTB epidemiology using antemortem tests in the North West, Adamawa, North and Extreme North Regions (115; 77; 120). Prevalences estimates in general have been higher when using tuberculin tests (0.18-10.6%) depending upon method and diagnostic cut-off used. Non-tuberculous mycobacteria (NTM) reactions were recorded in all studies. One study reported much higher prevalence estimates using a serological assay (29.75-43.24%)(120)) compared to reported tuberculin test prevalences (115; 77; 120). Hence differences in study design and diagnostic test performance make it difficult to compare prevalence estimates between studies.

Risk factors for bTB positivity, using the SCITT, have also been investigated in the North West and Adamawa Regions of Cameroon (115). Cattle in semi-intensive and beef systems appeared are at increased risk of being bTB positive. Risk of being bTB positive was also higher in older cattle than younger cattle. Cattle in smaller herds and cattle of the Gudali breed were reported to be at lower risk of being bTB positive. Although the roles of husbandry practices and wildlife in bTB transmission have not been investigated cattle. Previous risk factor analysis has not investigated the complexity of multiple risk factors for being bTB positive and only looked at individual risk factors. Hence the potential risk factors for bTB and potential routes for *M. bovis* transmission within cattle populations in Cameroon remain unclear.

1.2.5 Control, treatment and vaccination

Cameroon is 1 of only 7 SSA countries (n=49) that conduct some form of bTB surveillance and bTB control is primarily aimed at protecting public health through meat inspection. However none of the countries report national or regional prevalence estimates on an annual basis or have national cattle control strategies in place (22; 121).

In high-income countries, cattle control strategies against *M. bovis* are primarily in place to prevent zoonotic transmission (122; 123; 124). In most high-income countries bTB has been controlled to relatively low levels with an integrated sustained approach, of various types of control measure, being vital to minimise zoonotic transmission (45). For example in Australia 40+ years of bTB control measures facilitated eradication of *M. bovis* from the cattle population (122).

Public health control measures

In countries where milk production is unregulated and bTB is endemic, there is a possibility that human consumption of fresh milk may lead to zoonotic transmission of *M. bovis* (25). Increasing awareness of bTB can highlight the need for food safety control measures to prevent zoonotic transmission of *M. bovis* where national food chain legislation, or enforcement of, is absent (41). For example increasing awareness of the risk of *M. bovis* transmission from drinking fresh milk could help limit zoonotic transmission of *M. bovis* (54). Milk production in Cameroon is minimally regulated and bTB awareness is unknown in herdsmen. High awareness has been reported in abattoir workers (81%) although nearly a third (27%) consumed raw milk or meat (120). It is unclear if habits of abattoir workers reflect the consumption habits of herdsmen or their families who may also be at risk of zTB. Even one or two

infected cattle within a herd of 100+, where milk is pooled, can pose a public health risk to those consuming un-treated milk (60) hence treatment of milk is paramount in endemic disease settings. Heating of milk can destroy *M. bovis* organisms, limiting the risk of zoonotic transmission. In high-income countries it is compulsory to heat milk prior resale to minimise infectious disease transmission. This is commonly achieved by heating milk to 72° C for 15-60 seconds using industrial pasteurisation techniques. Destruction of *M. bovis* can also be achieved by heating milk at 63° C for 30 minutes (27). Soured milk is consumed in some SSA countries however destruction of *M. bovis* is not guaranteed (125). In most high-income countries this is regulated at national level and undertaken on an industrialised scale known as pasteurisation (27). In Cameroon milk resale and treatment is unregulated. Presumably milk is commonly consumed by cattle keepers in Cameroon, although the frequency of heating or souring treatment is unclear. Post-mortem inspection of slaughtered cattle to identify infected carcasses can prevent meat with lesions from entering the food chain (41; 123). Meat inspection does occur in Cameroon in all slaughter facilities in Cameroon including local slaughter slabs in small towns to larger municipal abattoirs in cities. Although results are used for government surveillance of bTB, use of this control measure alone will not minimise the risk of *M. bovis* transmission from live cattle.

Bovine control measures

Complete depopulation strategies, of potentially infected cattle or wildlife, is unsuitable control measure in most settings (122; 126). However targeted test and slaughter programs are the mainstay of bTB control globally (45). The basis of test and slaughter is through detection and removal of test positive or "reactor" animals with periodical retesting of animals (41). A reactor is an animal that has responded as

positive to bTB when using an ante-mortem test such as the skin tests and are removed with the aim to to reduce transmission of *M. bovis* (46). Such programs have been used from individual herd level to national level being the basis of international control to facilitate international trade of cattle and in some instances in the eradication of bTB (127). Success of such test and slaughter programs requires tracing all the cattle population and sustained re-testing in order to maintain control or eradication. For example in the UK a reduction in bTB prevalence from 40% in the 1930s to less than 1% over in the 1970s (31). To maintain a prevalence of less than 1%, thus control of bTB, sustained testing and surveillance of bTB has been required until present day.

Cameroonian cattle movements are in general poorly monitored and it is difficult to control infectious disease transmission. Unlike most high-income countries, such as the UK and New Zealand, where cattle movements are tracked using a database which has supported test and slaughter control programs (41; 76; 128). Subsequently test and slaughter programs would be unsustainable in pastoral systems in Cameroon.

Treatment and vaccination

Efficacious antimicrobials are available for treatment of human tuberculosis however their use in cattle is restricted, in part to minimise the development of antimicrobial resistance (18). Currently resistance is reported globally in human and zTB infections and untreated cases have a high mortality (129). Other factors include the cost of prolonged antimicrobial courses against the economics of production (31). Vaccination has been useful in the protection against *M. tuberculosis* and *M. bovis* infections in humans although its protection can be variable (130). The *Mycobacterium bovis* Bacille Calmette-Guerin (BCG) vaccine was developed from a

strain of *M. bovis* that was first cultured in the 1921 (131). The vaccine has been trialled in cattle with variable cross-protection (132). However standard bTB tests cannot distinguish vaccinated and infected individuals, hence would hinder test and slaughter control programs (131). Additionally an oral vaccine is being trialled in some wildlife species to limit *M. bovis* transmission to cattle where slaughter of wildlife is unacceptable (133).

1.2.6 Diagnosis

A gold standard diagnostic test is a test that most accurately defines the true status of the individual tested (134). There are a range of post-mortem and ante-mortem diagnostic tests for bTB. Some of which detect the organism *M. bovis* or aspects of the pathological and immunological responses to infection. Ideally a gold standard diagnostic test for bTB would have 100% sensitivity and specificity, however like many other infectious diseases there is no definitive gold standard diagnostic test for bTB (123; 78). In the absence of gold standard diagnostic test, the estimated performance of a specific diagnostic test is dependent to the diagnostic test it is compared against. Furthermore depending on what aspect of bTB the diagnostic test detects, the interpretation of the result will depend upon whether presence of infection or disease is most important to the user.

Postmortem diagnostics

Development of gross tubercles in infected tissues facilitates post-mortem detection of bTB relatively easily. Abattoir detection of TB lesions through meat inspection has been used for over 100 years as a method of passive surveillance and control for bTB. The practice was first instituted to protect the public from consumption of TB infected

meat in the UK in the early part of the 1900s (67). The location of TB lesions can also be used to investigate the route of infection in epidemiological studies, for example respiratory transmission was implicated where 70-90% of bTB lesions were detected in the lymph nodes of the head or thorax of infected carcasses (41; 46). False positive diagnosis of TB lesions is negligible, especially in high prevalence areas or with histopathological confirmation, but is dependent upon public health workers inspecting the carcass in a thorough manner (135). Abattoir detection rates of TB lesions vary greatly, ranging between 47-95% when compared with detailed PME, and sensitivity has been quoted be be as low as 28.5% (135; 41; 136). Gross TB lesions do not always develop in animals that test positive to antemortem bTB diagnostics (137). Between 50-80% of animals with no gross TB lesions react to other diagnostic tests such as the intradermal skin tests (135; 138; 139; 48). Absence of gross lesions may be due to early stages of infection, as lesions may take months or years to develop, and/ or differences in virulence of *M. bovis* genotypes (17; 140; 3). Today in many high-income countries with a low bTB prevalence, means that bTB lesions are infrequently as few chronically infected animals are present due to long-term test and slaughter control programs which remove "reactor" animals (46). Differences in *M. bovis* isolate virulence has been demonstrated where different genotypes have different abilities to infect, survive, multiply and cause bTB within the host (141). For example cattle infected with *M. bovis* genotype 3.140 was less likely to cause bTB pathology than other genotypes and maybe useful in future vaccine development (142).

In one study 10% of non-visible lesion reactor animals were shown to be infected with *M. bovis* via bacteriology (135). Where laboratory facilities are available identified TB Lesions are often confirmed with *M. bovis* culture and further genotyping to improve sensitivity. *Mycobacteria* species grow slowly usually

between 1 to 14 weeks on solid Lowenstein-Jensen media (LJ Media) (143). *M.tuberculosis* and *M. bovis* can be differentiated on LJ media by the addition of pyruvate which favours *M. bovis* growth. *Mycobacteria* speciesl culture is often mistaken to be the gold standard for bTB diagnosis. However culture has variable sensitivity and specificity for bTB diagnosis even with use of additional mycobacterium biochemical tests on isolates (138). Also microscopy of cultures can differentiate no further than MTC. Culture on solid media for bTB diagnosis is very slow. Liquid culture can be much quicker taking from 4 days to 3 weeks for mycobacterial growth, but requires specialist electronic culture appliances such as the mycobacteria growth indicator tube (MGIT) system (144; 138). Sensitivity of culture depends upon the method used, sterility of lesion collection and quality of lesion storage (135; 48). Due to culture and microscopy having low power to differentiate between members of the MTC, further genetic assays can be used to improve sensitivity of both lesion detection, or differentiation of isolate genotypes (145).

From sampled PME lesions genotyping is also useful in epidemiological studies describing the geographical distribution and transmission of *M. bovis* in cattle and other host species populations (146). Various genomic methods have been used to describe variation in *M. bovis* such as partial and whole genome methods (2; 147; 3). Partial genome methods include detecting tandem repeated sequences (e.g. Multilocus Variable Number Tandem Repeat (MIRU-VNTR) and IS6110 amplification), non-tandem repeated sequences (e.g. Spoligotyping), random repeated sequences (e.g. random amplified polymorphic deoxyribonucleic acid (RAPD) analysis), specific genes (e.g. multilocus sequence typing (MLST)), regions of difference (e.g. direct repeats (DR) typing) and single nucleotide polymorphism (SNP) typing (12). Spoligotyping, historically the most commonly used method, looks at spacer polymorphisms and deletions in the direct repeat region of the

chromosome and a database of known spoligotypes has been compiled attempting to catalogue *M. bovis* strain variation (3). Other genotyping methods have been used in addition to further describe *M. bovis* epidemiology at regional level. In Cameroon, a study by Njanpop-Laforcade describes how such techniques as restriction fragment length polymorphism (RFLP) along with spoligotyping can be used to describe regional distribution of *M. bovis* strains (113). Similar surveillance techniques have been achieved at herd level in the UK coupled with GIS mapping to describe bTB transmission between herds (148; 149; 146). West African *M. bovis* spoligotypes in Mali are related but distinct from the Cameroon spoligotypes with the additional loss of spacer 6 as well as spacer 30. It is postulated from genotyping studies that European cattle, initially imported to Cameroon, transmitted *M. bovis* to cattle destined for Mali. Due to localised cattle trade the Malian spoligotype strain has evolved to be distinct from Cameroonian strains (10; 117; 150). Hence *M. bovis* transmission routes and selection pressure on the pathogen can influence spoligotype patterns (3).

With the advent over the past decade of full genome sequencing there are now examples of the *M. bovis* sequence (151). Whole genome sequences will allow further understanding of the epidemiology and genetic diversity of *M. bovis* (12). Methods such as restriction endonuclease analysis (REA), RFLP, pulsed-field gel electrophoresis (PFGE), whole genome sequencing and microarray can be used. Whole genome sequencing techniques have also allowed identification of genetic differences between *M. bovis* and BCG vaccination strains (147; 152; 153). Such differentiation allows identification of further *M. bovis* specific antigens for diagnostic test development. Detection of specific antigens has enabled development of diagnostic tests that can differentiate between vaccinated and non-vaccinated cattle (The distinguish vaccinated from infected animals or "DIVA" diagnostic tests) (154).

Such tests could faciliate the use of BCG vaccination in bTB control strategies (131; 155; 156). However despite incomplete description of the *M. bovis* genotype using spoligotyping and other methods, these methods are still used as *M. bovis* genotype descriptors. This is due to their rapid analysis of isolates, low cost and ease of these methods when compared to full genome sequencing of *M. bovis*.

Worryingly, in many low-income countries meat inspection may be the only bTB control method implemented, mainly due to the relatively low cost of meat inspection, logistic problems with live animal testing and inadequate funds for culling or compensation programs (22; 48). Additionally, in low-income countries including much of SSA, quality of meat inspection training varies between abattoirs and countries (157; 158). In some low-income countries slaughter slabs and slaughter at home may contribute to human infections where meat is not inspected. Bacteriology and subsequent genomic assays are also of limited use in many situations due to long distances to laboratories and frequent power cuts. Maintenance of a cold chain for sampled bTB lesions is essential for bacteriology and inadequate sample storage leads to an increased number of contaminants and false negative results in cultures (108; 35; 138; 10). Overall PME, microbiological and genotyping assays are of useful in epidemiological and genetic studies, but of limited value in live animal surveillance studies. Hence immunological diagnostic tests remain vital for bTB epidemiological studies and control programs involving live cattle.

Antemortem diagnostics

Surveillance and control programs rely upon the accuracy of diagnostic tests implemented. Antemortem diagnostics are mainly related to detecting the immune response to *M. bovis* (Figure 1.2.6). Due to the early development of the Th1

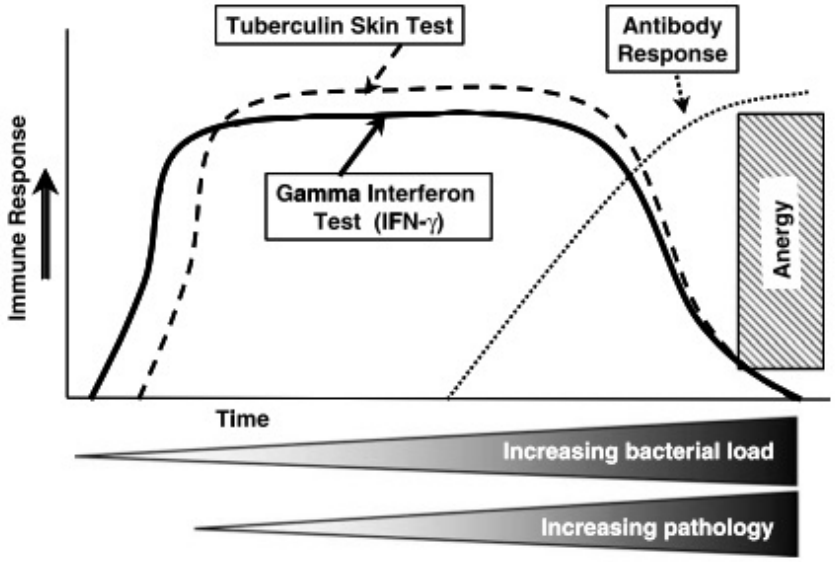


Figure 1.2: **A graphical representation of the immune responses detected by the various bTB diagnostic tests.**
(Reproduced with permission of the authors (78)).

responses promoting further CMI responses to *M. bovis* infection, assays that focus on detecting CMI for diagnosis are utilised worldwide (48). This is because they are more sensitive and detect infection earlier, which is essential for control programs. The two main CMI-based tests currently in use and approved by the OIE, are the intradermal tuberculin test in cattle and the laboratory-based interferon-gamma (IFN-γ assay. Both these assays detect the CMI response to *M. bovis* using different methods: the intradermal tuberculin test via delayed-type hypersensitivity and the IFN-γ assay via whole-blood cultures stimulated with tuberculin and assessed by enzyme-linked immunosorbent assay (ELISA) (159; 160). These tests are reliant upon the use of tuberculin, a crude mixture of protein based antigens initially developed from cultures of *M. bovis*, stimulating an immune response in animals with previous exposure to *M. bovis* (161). Tuberculin was initially developed for use as a human vaccine in the early 1900s later being developed in the 1920-1950s as part of diagnostics for both animal and human tuberculosis (78; 162; 67; 160). The tuberculin has been refined since then and has been developed into a purified protein

derivative (PPD) from a strain of *M. bovis*, AN5 from England in 1948 that is still used worldwide today (163; 48). Sensitivity and specificity for a particular diagnostic test are generally regarded as fixed in different populations when the identical test protocol is used (134). However bTB antemortem diagnostics that assess CMI have variable accuracy in different populations due to the stage of the disease, ubiquity of cross-reacting environmental mycobacteria and various other factors (78). These factors will also affect bTB diagnostic test predictive values; hence the disease dynamics of bTB continue to present many challenges for bTB diagnostic tests in cattle.

The intradermal tuberculin test was the first test developed in British cattle as part of bTB test and slaughter programs in the early 20th century, becoming compulsory in the 1950s in addition to abattoir detection of lesions, to protect public health. Subsequently the intradermal tuberculin test has become the OIE international standard *M. bovis* diagnostic tool for bTB control. In its simplest form the test relies upon intradermal injection of tuberculin/PPD and then the type IV hypersensitivity response, visualised by a swelling of the skin, being measured approximately 72 hours later (164). A hypersensitivity response implies exposure to *M. bovis*. This response is usually measured by palpation or calliper measurement of the skin. Type IV hypersensitivity responses are due to sensitisation of T cells weeks after *M. bovis* infection (78; 140; 48). Where a single injection is performed, in the cervical region or caudal fold of the tail, the test is regarded as the single intradermal skin test (SIT) (163; 46; 160). The SIT is conducted in various countries where prevalences of bTB are low, such as in USA and New Zealand, and has been used successfully in bTB eradication schemes in Australia (163; 57). Sensitivities vary between 68-96.8% within different regions of the world where the SIT is routinely conducted (165; 48). Specificity also varies to a lesser extent (75.5-98.8%) but is significantly reduced in

some geographical regions due to ubiquity of cross-reacting NTM. This is particularly true in areas where cattle are reared extensively due to the presence of mycobacteria in the soil. For example in the UK where 6-12% of SIT reactions were regarded as false positives and in Africa where there are numerous environmental mycobacteria in pastoral farming systems (82; 77; 109). To attempt to overcome this problem the SCITT was developed where the inflammatory reaction to injection of avian PPD, regarded as exposure to non-*M. bovis* mycobacteria, is compared to the reaction to the bovine PPD at 72 hours post-injection. This is in concordance with OIE guidelines or country specific control policies (165). Avian PPD is used to represent reaction to NTMs and compared to the bovine PPD to assess for NTM cross-reaction (166). The SCITT has an increased specificity (88.8-100%), when compared to the SIT and reduces the proportion of false positives attributed to cross-reacting mycobacteria which is particularly important in control programs. As specificity and sensitivity have an inverse relationship, a moderate decline in sensitivity, 55.1-93.5%, is noted with the SCITT (48). Reduced sensitivity results in more false negative animals and this is a problem at the start of eradication programs in regions where prevalence of bTB in high.

Development of another CMI test, IFN-γ assay, was a breakthrough as the test has reported higher sensitivity than the intradermal tuberculin tests, detecting infected animals 1 to 4 weeks post infection (163). It was first developed in the 1980s to be used in the Australian bTB eradication program but subsequently has been used as an ancillary bTB test internationally (159; 167). The test primarily detects IFN-γ a cytokine which is produced from T lymphocytes within 16-24 hours of *M. bovis* infection within macrophages (168; 161). The assay is comparative, like the SCITT, assessing IFN-γ production when whole blood cultures are exposed to various antigens (avian PPD, bovine PPD and a negative control). Cultures are incubated for

16-24 hours at 37°C, the plasma supernatants are extracted and can be stored between 4°C and -20°C if required to be used in an IFN-γ ELISA assay, commercially marketed as Bovigam®. Comparison of the optical densities (OD) of the plasma exposed to avian and bovine PPD's are used to determine *M. bovis* infection status, an animal likely to be positive with greater IFN-γ production with the bovine PPD than avian PPD in whole blood cultures. The OIE have designated the test to be useful as an alternative test for international trade, and it is used in many developed countries as an ancillary assay to the skin test in test and slaughter strategies (48). Sensitivities range between 73-100% (Median: 87.6%) and specificities of the IFN-γ assay range between 85-99.6% (Median: 96.6%) (78). Like the intradermal skin test, accuracy is dependent upon the cattle population tested, with for example specificity being reduced in populations where exposure to environmental mycobacteria occurs. Despite the vast use of CMI diagnostics, neither CMI test, used individually or in combination, has been shown to be an adequate gold standard for bTB diagnosis in a field situation. This is largely due to significant proportions of false positives and false negatives with CMI diagnostics in naturally-infected cattle populations.

Causes for false positive and negatives can be broadly divided into three types, the first concerning host-related-factors. Upon initial *M. bovis* infection animals may not react to the skin test for up to 1 to 9 weeks afterwards; being called the pre-allergic phase (165). At the other extreme, animals with generalised or overwhelming bTB also may not produce a CMI response, termed anergy. Anergic animals may be detected to some extent by serological assays (169; 78). Temporary desensitisation to subsequent diagnostic tests can occur from 3-60 days post injection of tuberculin/ PPD (165; 170) hence if a second skin test is used to confirm an animal's bTB status a waiting of 60 days period is required (78). The length of waiting period to repeat the skin test is dependent upon country specific legislation and in some countries an

alternative diagnostic test is used such as the IFN-γ assay. Previous skin testing has also been shown to reduce the response to bovine PPD in the IFN-γ assay but in the majority of studies the response is increased with the SIT or not at all with the SCITT. One advantage with using the IFN-γ assay alone is that it can be repeated with no waiting period as the immune stimulation is in vitro. Immunosuppressed animals, such as stressed individuals or those administered immunosuppressive drugs (e.g. corticosteriods), have reduced CMI responses (85). Certain co-infections, such as *Fasciola* species, may also contribute to false negative results due to their active manipulation of host CMI responses (78; 171) (Discussed further in section 1.4.3). Exposure to other mycobacteria such as other tuberculous mycobacteria (e.g. *M. tuberculosis*), other pathogenic mycobacteria (e.g. *Mycobacterium avium* subspecies *paratuberculosis* which causes Johnes Disease)) and NTM (e.g. *M. kansasii*) have been shown to increase the avian reaction with CMI diagnostics (166; 4). Similarly age-related factors may increase false positive results, for example when the IFN-γ assay is used in cattle less than 6 months old cattle due to cross-reactions with innate immune system responses (172; 86).

Secondly, factors related to the tuberculins, used in either type of CMI test, affect diagnostic sensitivity. Worldwide the potency of batches of bovine PPD is assessed in experimental studies in guinea pigs and cattle. Various manufacturers produce PPDs internationally; hence brands used will vary between studies and countries (173; 174). Potency tests appear to vary between batches but also between brands (48). International standards dictate that the minimum dose of 2000IU, for avian and bovine PPD, and a recent study showed that many of the commercially available PPDs would not reach this international standard (173). Additionally corresponding avian PPD potency varied between brands; overall markedly affecting bTB diagnosis (175). Improvements in specificity however have been achieved with use of defined

antigens identified through genomic studies singly or as cocktails (164). Proteins such as ESAT-6, CFP-10 and additional cocktails have been used to achieve near 100% specificity in skin and IFN-γ tests (176; 177). In particular ESAT-6 appears to be most specific for *M. bovis* infections, as the ESAT-6 gene is only present in *M. bovis* and *M.tuberculosis* (85; 167). Protein cocktails also appear to be useful in improving specificity due to individual animal variability of antigen recognition, including in cattle which are likely to encounter a variety of NTMs such as those reared in extensive pastoral systems in SSA (178; 179). However with use of more specific antigens there were general decreases in sensitivity This can be addressed through altering diagnostic cut-off values (172; 48).

Thirdly factors associated with human error when conducting the tests can affect accuracy of CMI diagnostics. Human error is particularly important when different operatives conduct the skin tests. This is because measurement of skin thickness will vary slightly between operatives depending upon how much pressure they put on the callipers used to measure the skin thickness. As long as the the same specified person conducts the two stages of the test the differences should be negligible. Also procedural errors include subcutaneous, rather than intradermal, injection of tuberculin, equipment malfunctions, problems associated with field conditions and tester bias; possibly due to owner of the animal's being present (78). The latter is eliminated with the laboratory-based IFN-γ assay and laboratory quality assurance measures should minimise procedural errors. However transit time from the field to the laboratory of blood samples can affect viability of blood cultures; along with maintenance of advised temperature of whole blood (10-26^{o}C) (85). This was of particular concern in Australia when the test was under development, but is also relevant for its use in a SSA setting (167). Ultimately logistics also hinder the use of the skin test in some settings due to the need for two visits.

The variable sensitivities and specificities of CMI diagnostics can be best utilised in different situations, depending on prevalence of bTB and reason for bTB testing. As both skin testing and IFN-γ assess CMI; unsurprisingly they detect distinct yet overlapping groups of bTB infected individuals in a population (180; 181; 182). Therefore using the tests in combination can improve sensitivity of bTB diagnosis. With skin testing historically being used as the primary test and IFN-γ assay as an ancillary test; detecting bTB positive or negative animals can be maximised by using the tests in parallel. Parallel testing has been used in New Zealand, where skin testing and IFN-γ assay are used in quick succession, to identify as many false negatives to the skin test as possible. Parallel testing, therefore enhances bTB diagnostic sensitivity and is used as part of new outbreak or pre-movement bTB testing in New Zealand (163). Serial testing has also been utilised, where IFN-γ testing occurs after initial result of the skin test, to improve specificity. For example where wildlife vectors are of concern or environmental mycobacteria are ubiquitous (78). In epidemiological studies combinations of serological and CMI tests have also been used to increase accuracy of bTB prevalence in a population; capturing animals at different stages of the immune response (75; 35; 183). Both the skin tests and the IFN-γ assay measure continuous variables and require cut-off values to be set to determine whether an animal is positive, negative or inconclusive (unclear results). These can be calculated in various ways depending upon the test and the population being sampled. For example in SCITT is interpreted at "Standard" (>4mm) or "Severe" (>2mm) cut-off values in the UK, dependent upon the prevalence of bTB within a particular country or scenario. "Severe" cut-off values allow for a smaller increase in skin thickness than "Standard" cut-offs; therefore increasing sensitivity. In areas where there is a high prevalence of bTB "Severe" interpretation is used to increase sensitivity and minimise the number of false negatives as part of the national eradication program (46). Performance of the skin test does vary in different

environments and hence standard cut-off values recommended by the OIE may not be suitable in different geographical locations. This is true in Africa where different environmental mycobacteria are present and *Bos indicus* cattle predominate rather than *Bos taurus* cattle (97; 35; 120). Hence variable cut-off allow maximal test performance in environments where multiple factors affect diagnostic results. Cut-off values may also be changed or test protocol modified when adapted for other species; such as recommendations for the SCITT in camelids where the skin is recommended to be re-measured at 120 hours rather than 72 hours post PPD inoculation (184; 80). Cut-off values can also be changed for the IFN-γ assay, again at "Standard" ($\geq$0.1) and "Severe" ($\geq$0.05) interpretations as has been successfully been used in France as part of an eradication program (185). By lowering the positive cut-off value of the IFN-γ assay to "Severe" interpretation ($\geq$0.05), the sensitivity of the assay could be maximised to 93.0% where breakdowns occurred (Specificity: 71.8%). In low risk areas specificity could be maximised to a 94.3% (Sensitivity: 77.0%); ultimately leading to bTB control to allow international cattle trade. In the absence of a gold standard diagnostic for bTB; Bayesian models appear promising to assess test performance at changeable diagnostic cut-offs in different populations and ultimately more accurate assessment of *M. bovis* prevalence. For example in Spain IFN-γ assay cut-offs were changed depending upon the risk area and even breed of cattle tested (186). By using a Bayesian non-gold standard model a cut-off value with appropriate sensitivity could be selected for both skin testing and IFN-γ assay in the Spanish control program. Thus the model enabled adaptation of the bTB diagnostics cut-off values, either to maximise sensitivity or specificity, depending upon bTB prevalence and logistic challenges of the control program. Such as using the IFN-γ assay where there was a difficulty in performing skin testing in fighting bulls.

As with many infectious diseases humoral immune responses, a major component of

the Th2 immune response, are measured to assess exposure and infection to a particular organism. This is also true for *M. bovis* infection and usually develop with increasing bacterial load in later stages of *M. bovis* infection (161; 140). Antibody (IgG1) responses to *M. bovis* have been detected as early as 2 weeks to 2 months post infection in experimental studies but more commonly from 90-100 days post challenge (48; 187). Antibody production is often associated with chronic stages of infection and generalised TB where CMI responses are waning (See Figure 1.2.6) (78; 46). Hence this subset of *M. bovis* infected animals are referred to as anergic; often being highly infectious due to high burdens of *M. bovis*. Therefore serological tests have been of interest for diagnosis of anergic and chronically diseased animals; with a variety of techniques developed for antibody detection (48). They also tend to be easy and rapid to perform; for example lateral flow/ "penside" formats are favourable in the field such as with cattle movements at country boarders, in low-resource countries and in wildlife populations (188; 189; 187; 190). They have also been used as herd tests on bulk milk tank samples (191). Serological tests tend to suffer from variable sensitivity (60.0-96.0%) due to cattle developing of serological responses inconsistently post-infection (187). Cocktails of antigens have been added to some tests to improve sensitivity of antibody based tests (77.0-94.8%) to distinguish specific antibodies to *M. bovis* from other *Mycobacteria* (192; 183; 193). Skin testing, from 60 days previous, can also boost serological responses (46). Serological diagnosis, depending on the serological assay used, is linked to stage and severity of infection with the change in dominance of Th1 to Th2 immune response (161). Ultimately, due to later production of a humoral response in the course of infection, serological tests have a high proportion of false negatives when compared to CMI diagnostics. Hence the CMI is predominately utilised for early detection of *M. bovis* infection.

Despite the many advances in bTB diagnosis and the variety of bTB diagnostic tests none are 100% sensitive or specific. Hence, no gold standard diagnostic test is currently available for diagnosis of bTB. *M. bovis* infected animals continue to evade current diagnostic tests resulting in unacceptable numbers of false negative test results. Due to the chronic time scale of bTB disease, there maybe other factors that may influence the immune response to bTB. Such factors will ultimately affect diagnostic test performance in an endemic disease setting such as Cameroon and should be explored further. For example further understanding of the role of co-infections that might altering the bovine immune response associated in detecting *M. bovis* infection, such as *Fasciola* species, may go someway to explaining discrepancies in bTB diagnosis.

1.3 Bovine *Fasciola* infections

1.3.1 Significance and parasite biology

Fasciola species infections have been reported in cattle populations in every continent other than Antartica. Although many herbivorous species can be infected with the parasite, including man (194), infections in ruminants such as cattle, buffalo, sheep and goats are most commonly reported (195; 196). Ruminant infections account for $3 billion of production losses per annum globally, although the impact on sub-Saharan African cattle populations is undefined (197; 198). Production losses are related to the *Fasciola* specie's complex life cycles and subsequent disease termed fasciolosis. There are 2 species of *Fasciola* that infect cattle; *Fasciola hepatica* often found in temperate climates although is also found in sub-tropical climates. *Fasciola gigantica* is mainly found in tropical climates such as sub-Saharan Africa and parts of

Asia (199). The 2 species can breed to form hybrid intermediate species in some areas where their distribution overlaps (200). The distribution of the 2 species is governed by the presence of intermediate host species that are integral and specific to *Fasciola* species life cycles (Figure 1.3.1).

Adult stage of the parasite mature in the bile ducts of infected cattle and release eggs (4000-25,000+ per parasite per day (201; 202)) into the bile these are shed via the small intestine into the faeces and subsequently the environment. At pasture embroynated eggs take 2-6 weeks to hatch as a miracidium which is dependent for temperatures above 10° C. This ciliated form of the parasite is mobile in water and within 20-30 hours it must find a suitable intermediate snail host to infect (203). Primarily aquatic snails are the parasites intermediate host, such as *Radix natalensis* in sub-Saharan Africa and *Radix rufescens* in parts of Asia, from the Radix auricularia complex. Snail species will may vary within different localities of the same country. *Radix natalensis* species in many parts of Africa occur $\leq$2000 meters above mean sea level (mamsl). The preferred host for *Fasciola hepatica* is the mud snail, *Galba truncatula*, yet can occasionally infect other snail species $\geq$4100mamsl (204). The distribution of the parasite is governed by the lifecycle and distribution of its snail intermediate hosts (205). The miracidium penetrates the snail, develops into a sporocyst stage and migrates to the digestive gland of the snail. Subsequently the parasite multiplies through a redia stage to be shed from the snail intermittently as motile cercariae from 2 months post-infection (202). Within a few minutes motile cercaria attach to plants or hard surfaces and encyst to form infective metacercaria. Susceptible definitive hosts are infected through consuming metacercaria. It is unclear how long metacercaria can remain viable at pasture but they have been reported to remain viable for up to 23 weeks at temperatures $\leq$35° C (206). Furthermore with *F. gigantica* can detach from plant material to float in water which

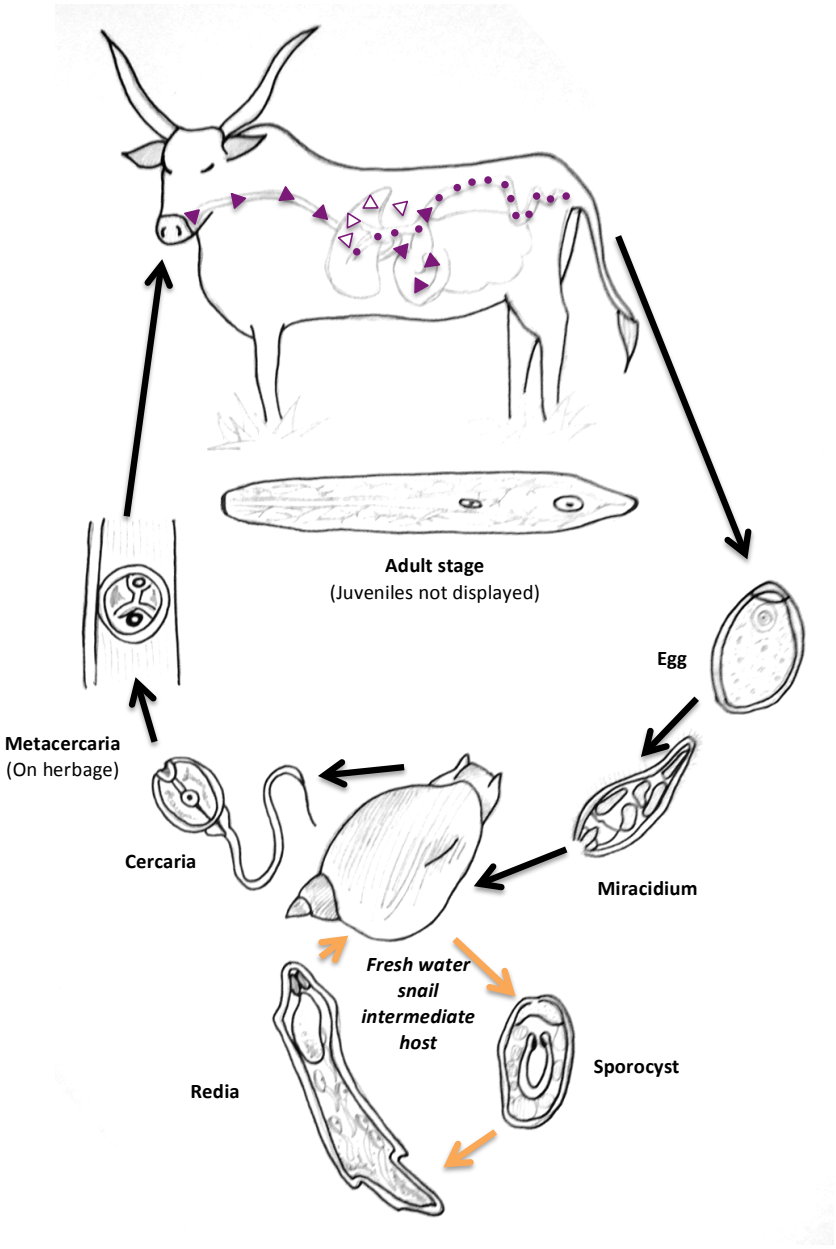


Figure 1.3: **A graphical representation of the life cycle of *Fasciola gigantica*.** Black: external lifecycle; Orange: intermediate host lifecycle with an example species of intermediate host (*Radix natalensis*); Purple: definitive host lifecycle; Purple triangles: metacercaria upon ingestion; White triangles: migrating juveniles to adults in the bile ducts; Purple circles: eggs deposited by adults in the bile ducts passed via the faeces into the environment.

may aid spread of infection (203). Once in the host metacercaria hatch and penetrate the intestinal mucosa into the peritoneum then migrate towards the liver (207). Juvenile stages migrate through the liver, digesting their way through by secretion of excretory/ secretory products (ESP) including various cathepsin enzymes (208), until they reach the bile ducts to sexually mature into adults. Reproduction is sexual, as adults are hermaphrodite, with a pre-patent period for 91-112 days with *F. gigantica* and 70-84 days for *F. hepatica* bovine infection (199). Adult *F. gigantica* parasites have been reported to survive for 3-4 years within the host (209).

Subsequent gross liver pathology is mainly related to the presence of adults within the bile ducts in cattle. Adults within the bile ducts cause hyperplastic cholangitis and are easily identifiable at PME. In cattle hypertrophy of the ducts can lead to calcification of the ducts (210). Pathology caused migrating juveniles being a minor finding although haemorrhagic and necrotic tracks can be seen on the cut surface of the liver. Heavy burdens, particularly with *F. gigantica*, can lead to hypertrophy and fibrosis of the liver. Although pathological changes are usually permanent in light infections it has been reported that pathology is reversible in the absence of further challenge (203). Pathological changes in cattle usually lead to sub-clinical disease which results in production losses such as weight loss and drop in milk production (210). Clinical disease is related to the migration of the parasites within the definitive host presenting as weight loss, sub-mandibular oedema and anaemia. Diarrhoea can also be present on initial infection from parasite penetration of the intestinal mucosa (211).

1.3.2 Epidemiology and control in Cameroon

Historically bovine infections in Cameroon were thought to be exclusively *F. gigantica* from morphological identification and infecting cattle throughout the

country (212; 198). *Fasciola gigantica* intermediate host species are suspected to be *R. natalensis*, which are found around permanent water bodies, and play a obligate role in parasite epidemiology. Schillhorn van Veen postulated that challenge was seasonal in pastoral herds in Central-West Africa (213). In the dry season when snail populations appeared at their highest cattle congregate around water bodies such as lakes and streams. In last couple of decades, infection is still reported to be endemic in cattle populations with $\geq$80% prevalence reported in a few localised abattoir surveys in cattle (214; 215) and small ruminant (216; 217) populations in the North and West of the country. Similarly adjacent Nigeria (218; 219; 220; 221; 222) only reports *F. gigantica* infections in cattle (determined by morphological identification). Although *F. gigantica* infection is the most common species in sub-Saharan Africa pockets of *F. hepatica* infection do exist where susceptible intermediate hosts are present (202). As intermediate species, formed from hybrids between the 2 parasites, may exist but would be morphologically indistinguishable and can only be differentiated by genomic methods (223). In Tanzania both species were identified along with hybrids between the two. The reason for the presence of the 2 species is postulated as being that these were introduced from imported cattle from temperate climates or possibly migratory birds distributed infected snail species onto the continent (205). Mixed *Fasciola* infections have also been reported in Niger which has cattle trade links with Cameroon (224). Hence despite *F. gigantica* only being reported infecting Cameroonian cattle few epidemiological surveys have been undertaken to define *Fasciola* species present.

Control of *Fasciola* infection is focused on prevention of access to snail habitats and strategic use of anthelmintic treatments (Flukicides). Prevention of access of contaminated pasture is difficult in endemic settings, such as Cameroon, where access to water for cattle from natural sources is synonymous with *F. gigantica* intermediate

host habits. Fencing off of contaminated areas and draining pasture have been used effectively to prevent infection in semi-intensive and intensive production systems. Molluscicides have been used but have gone out of favour due to their ecological impact (225). A variety of flukicides are available to treat *Fasciola* infections in cattle such as triclabendazole (12mg/kg), targeting juvenile and adult stages, and others which target specific stages such as closantel (5mg/kg), oxyclozanide (10mg/kg), nitroxynil (10/mg/kg) and rafoxanide (10mg/kg). Albendazole (10mg/kg) has limited efficacy against adult stages of *Fasciola* but also has nematode activity that maybe useful in tropical climates (203). Choice of different anthelmintics is likely to depend upon stages of parasite present, cost, dosing method and availability of types of anthelmintic. Excessive use of flukicides can lead to selection of an increased proportion of resistant genotypes of *Fasciola* within the parasite population. Resistance to flukicides has been reported such as triclabendazole in high income countries (226) with *F. hepatica* infections. Although albendazole resistance has been reported, in Tanzania(227) with *F. gigantica* infections, the validity of such reports remains debatable as in theory survival of susceptible juvenile parasites should dilute the effect of selection pressure on the adult parasite population. In some countries development of disease forecasting systems have improved targeted control of *Fasciola* infection and fasciolosis (228; 225; 229). In Cameroon control measures are often absent and efforts are focused around treating cases of fasciolosis rather than prevention. Partly due to limited resources and paucity of information on the epidemiology of infections.

1.3.3 Diagnosis

Clinical diagnosis of bovine *Fasciola* infections is difficult due to the majority of infections being subclinical and may not present as clinical fasciolosis. Measuring

serum hepatic enzymes (230), such as aspartate aminotransferase (Reference: 78-132 units/L) can be useful in the migratory phase of the parasite's lifecycle where these enzymes maybe elevated in peripheral blood. In chronic stages of infection, usually seen in cattle, L-gamma-glutamyl-transferase (Reference: 6.1-17.4 units/L) maybe elevated from baseline values due to its close association with the bile ducts. However sensitivity of biochemistry is likely to be variable at different stages of infection and none of the enzymes are specific for *Fasciola* infection (195; 211).

Post mortem examination is considered as a gold standard diagnostic for *Fasciola* infection, through examination of the bovine liver to identify characteristic pathology or parasites directly, yet is impossible in the live animal (231). Detailed PME, by slicing through 1cm segments of the liver, is used to quantify burden of parasites. Less detailed techniques can be used to make a diagnosis, by making 2 incisions at the level of the bile ducts, and is often used in abattoirs at meat inspection to assess quality for consumption (232). The specificity of the assay is 88<100% (233; 234) due to the pathognomonic nature of lesions. However sensitivity (63.2-68%) (233; 234) can be variable and will vary between abattoirs (233; 235; 215). Faecal worm egg detection or counts (FWEC) have historically been used to diagnose sub-clinical and clinical fasciolosis due to the tests high specificity (95-99%) where identification of one egg is classed as a positive (233; 234; 201; 226; 198). Morphological appearance of eggs is similar between *F. hepatica* and *F. gigantica*. The test is inappropriate to detect juvenile parasites which do not produce eggs in the first 8-12 weeks post infection (236; 198). The latter probably accounts for the variable sensitivity of the FWEC (30-90%) as well as intermittent shedding of eggs from the gall bladder into the faeces (237; 238; 239). In recent years the development of a commercial cathepsin copro-antigen ELISA has been used to detect *F. hepatica* antigen in the faeces of experimentally infected sheep from 4 weeks post-infection

(240; 239). Although the test is not fully validated in cattle it can detect early stages of pre-patent infections and subsequently is deemed a sensitive test (77-94%) depending upon the cut-off value used (234; 241; 239). Specificity of the test (93-99%) demonstrates there is little cross-reaction between antigens of other helminth infections (234; 241; 239). Recently the performance of a *F. gigantica* cathepsin copro-antigen was shown to have similar sensitivity (95%) and specificity (91%) in cattle however it is not commercially available (242). A *F. hepatica* cathepsin copro-antigen ELISA have been used to detect *F. gigantica* infections in sheep 3-6 weeks post-infection (240) but its performance has not been investigated in cattle.

Serological assays have been used to identify exposure to *F. gigantica* (197) and *F. hepatica* (226). Due to the predominant humoral responses produced with *Fasciola* infections and the ability to test large numbers of samples quickly. Serological methods are useful for testing presence of *Fasciola* infection at herd level in dairy cattle by bulk milk testing (243). Also measuring humoral response can be useful in monitoring the evolving epidemiology of *Fasciola* such as in disease forecasting systems (244; 245). Most serological assays are in the form of ELISA methods to detect antibodies in response to ESP antigens (237; 246) secreted from the parasite or fractions of these proteins (f2 antigens) (247). For assessing exposure to *F. hepatica* infections the ELISAs are sensitive as they detect pre-patent infections prior pathological changes (86-100%). Specificity (83-96%) can be compromised in the presence of other trematodes due to serological cross-reactions (226). Reported sensitivity and specificity of these assays can be much lower for diagnosing infection, compared to other assays, as the diagnostic cannot differentiate current from previous exposure (234; 239). Similar findings have been reported for *F. gigantica* assays however there are none that are commercially available. *Fasciola hepatica* ELISAs have been used to detect *F. gigantica* infections in cattle (222; 248; 249), however the

performance of *F.hepatica* ELISAs for *F. gigantica* diagnosis is likely to vary in populations. Especially where both species are present due to cross-reactions between species antigens (237; 250). Therefore it is important to identify *Fasciola* species within a population and select an appropriate ELISA technique.

1.4 Bovine tuberculosis and *Fasciola* co-infection

Pathogens infect multicellular organisms as part of their lifecycle to survive and multiply. When a naive host is infected with a pathogen, and is accessible to the host's immune system, the host will try to mount an immune response to eliminate the infection. Development of disease is due to an aspect of this immune response that is detrimental to the host's homeostasis (251). Development of a disease can be therefore influenced by the immunological response produced to a specific pathogen at different stages of its pathogenesis. Diagnosis of an infectious disease can be via direct detection of the specific pathogen or detection of the host's immune response to the pathogen (134). For example *Mycobacteria* species, like other intracellular pathogens, stimulates a strongly polarised cellular response mediated by the Th1 immunity (Figure 1.4) (252). Conversely in general; extracellular pathogens such as helminths stimulate a humoral or Th2 mediated response. Many helminths can evade and modulate host immune responses into a Th2 predominated responses which might be important for their balance between parasite survival and parasitic disease (253). Hence studies of mono-infections have progressed our understanding of how bacteria and helminths stimulate and interact differently with host immune responses.

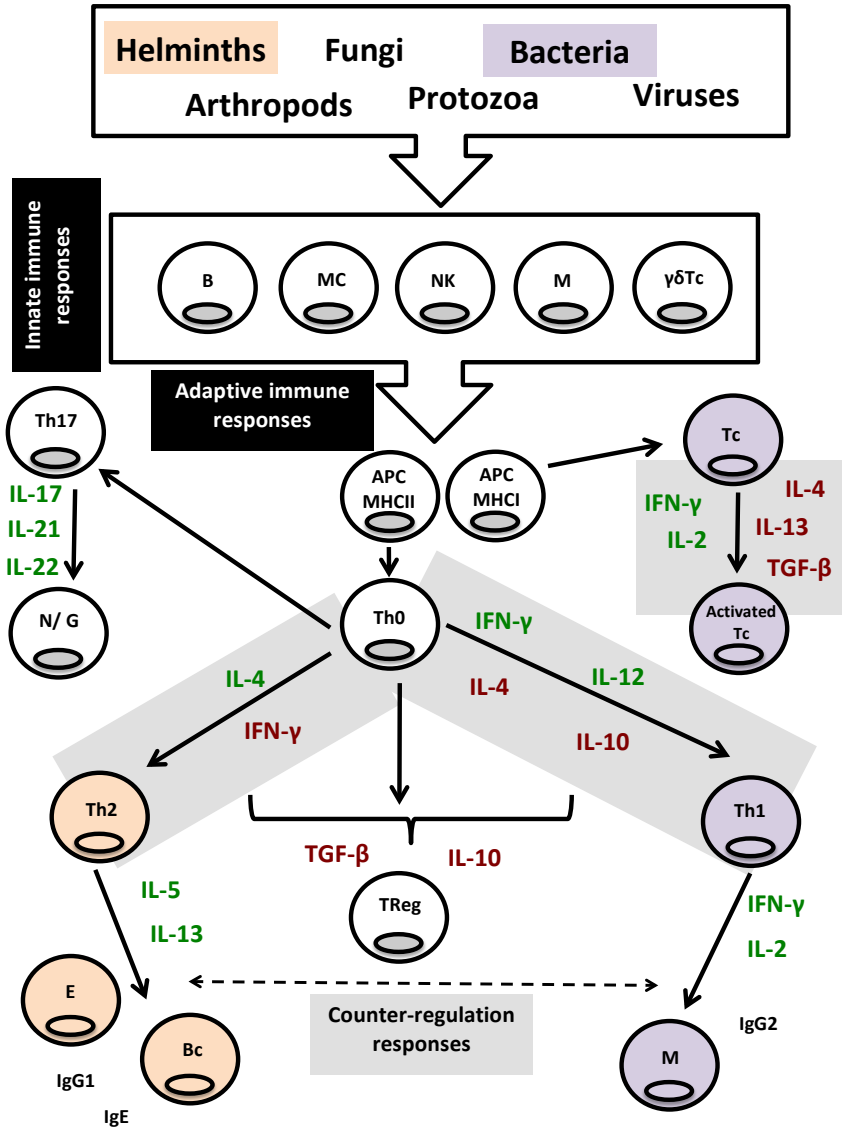


Figure 1.4: **A generic graphical representation of the predominant immune responses to bacteria and helminths in cattle.**
Adapted from (254; 203).Orange: Helminth predominant immune responses; Purple: Bacteria predominant immune responses; B: Basophil; MC: Mast cell; NK: Natural killer cell; M: Macrophage; Tc: T cell; APC: Antigen presenting cell; MHC: Major histocompatibility complex; Th: T helper cell; Treg: T regulatory cell; N: Neutrophil; G: Other granulocytes; Bc: B plasma cell. Green cytokines = Up-regulation of immune response. Red cytokines = Down-regulation of immune response.

1.4.1 Immunology and pathogenesis of bovine tuberculosis

Immune responses

Presentation of clinical disease is dependent upon extent of pathology (61) and therefore clinical signs are not always present with onset of immunological responses to *M. bovis* and are not specific to bTB. Therefore antemortem diagnosis of bTB often has to focus upon detection of *M. bovis* infection at the various stages of the immune response, as shown in figure 1.2.6 (161; 78; 48). *Mycobacterium bovis* is an intracellular pathogen that produces predominately CMI responses throughout the initial phases of infection (140; 17). Alveolar macrophages become infected with *M. bovis* through phagocytosis of bacilli upon infection (164). Macrophages retain *Mycobacteria* species within the phagosome for intracellular destruction however *Mycobacteria* species survive in this environment to reproduce and infect other macrophages and dendritic cells (DC) (131; 255). When initial infected macrophages die, $\gamma\delta$ T cells and natural killer (NK) cells are involved in initial innate immune responses towards progression of adaptive immune responses of mainly $CD4^+$ but also $CD8^+$ T cells (256; 257). Predominantly interleukin- (IL-) 12 and interferon-gamma (IFN-γ) responses initiate Th1 responses produced by $CD4^+$, $CD8^+$ T cells and NK cells (258; 259) (Figure 1.4). Interleukin-2 and IFN-γ cytokine responses can be detected 14 days post infection and such responses are important in continued predominance of the Th1 response with *M. bovis* infection (256; 260). Ultimately these Th1 responses predominate through these initial stages of infection although few longitudinal studies have been conducted to understand the dynamic of these responses in latency or development of clinical disease.

Th2 immune responses occur much later in the immune cascade often in the chronic

stages of disease from lesion development months or years after infection (140) leading to a rise in Th2 responses with a decrease in Th1 responses and severity in lesion size possibly related to limiting damage (258). B cells can be detected from 42 days post-lesion development and IgG1 has been associated with increased lesion development (261; 262). Humoral responses are more pronounced in cattle with systemic lesion distribution associated with chronic disease (256). However IgG1 antibody responses are not consistently detected in all infected cattle nor cattle in chronic stages of disease (46).

Lesion development

Development of white granulomatous lesions, often described as tubercles, in tissues is a result of this CMI response (137). These lesions develop with early domination $\gamma\delta$ T cells with their cytokine responses recruiting further macrophages for phagocytosis and further antigen presentation of *M. bovis* (257). Sensitised T cells and epithelioid cells coalesce within the granuloma mediated by Th1 cytokines (93; 161). Engulfment of infected macrophage and *M. bovis* debris by these cells leads to further granuloma development including recruitment of Langhan's giant cells, lymphocytes and fibrocytes encapsulate the granuloma (261; 70). Micro-lesions can develop from 7 days post *M. bovis* infection but gross-lesions, visible to the naked eye, are not detected until bacilli are multiplying rapidly by 14 days post-infection (263; 137). From 21 days lesions are often surrounded by macrophages with a necrotic centre made up of neutrophils and bacilli with fewer $\gamma\delta$ T cells (264; 257). From 41 days post infection the lesion is often encapsulated and the centre of the lesion may mineralise (261; 70). Depressed Th1 responses are often associated with development of disease and have been implicated with limiting development of extensive pathology and disease (258). Distribution of lesions is thought to be

influenced by the mode of transmission and development of the primary complex (138; 17). Aerosol transmission commonly results in lesions in lung tissue, bronchial and mediastinal LNs. Lymph nodes of the head are often also infected, such as the retropharyngeal and sub-maxillary LN, in absence of lung tissue and LN which might indicate earlier stages of aerosol and possibly oral infection (17). Oral transmission results in mesenteric LN infection which might be seen with large infective doses seen with drinking milk in calves (58). Either of these forms can lead to disseminated systemic disease involving multiple organ systems such as mammary, urogenital and hepatic tissues (61). Disseminated disease is not common in countries where bTB is controlled but is reported in endemic settings (26). Lesion distribution is not always consistent with route of infection (265; 137) hence other factors may also be involved in systemic dissemination of bacilli.

It is worth noting that laboratory studies have mostly investigated respiratory forms of transmission and most transmission models provide larger doses of infection than would be seen in natural infections. Lower doses in natural infections may develop immune responses and pathology differently at different doses of *M. bovis* (264). Commonly experimental infections instil doses of $1x10^3$-5 $x10^5$ colony forming units (cfu) intranasally (69; 70; 71) where as infections under natural conditions are likely to be smaller (92cfu) and still can shed *M. bovis* 100 days after infection (66). Furthermore a relationship between dose, lesion size and distribution does not always appear to be predictable with a range of pathological presentations possible (266).

1.4.2 Immunology and immunomodulation of *Fasciola* infections

Immune responses

T helper 1 immune responses, including production of IFN-γ can be detected 2-5 weeks post *Fasciola* infection in cattle (267; 268). Classically activated macrophages also act on the migrating stages of the parasite through nitric oxide and free-radical production (269; 270). However Th2 responses, such as IL-4 and IgG1 responses, tend to be the predominant immune responses developing from 24 hours post-infection in mice. Mice also show minimal IFN-γ IL-2 and IgG2 production by 3 weeks post-infection (271; 272). In bovine infections IgG1 antibodies are produced in response to ESP, somatic and tegument antigens from the parasite throughout infection (208). T helper 2 antibody responses predominate, peaking at 8-10 weeks post-infection, with minimal Th1 cytokine expression (268; 273). Subsequently mixed populations of eosinophils, leucocytes, macrophages and giant cells are typically detectable in liver pathology in sheep and cattle (274; 273). Chronic infections in cattle lead to hepatic fibrosis induced by deposition of Th2 induced immune-complexes leading to activated antibody dependent cell cytotoxicity (ADCC) with stimulation of alternatively activated macrophages (274; 198; 275). However Th 2 responses are not necessarily protective as infections can re-occur in cattle despite hepatic damage, fibrosis and calcification of the bile ducts (276; 277). Subsequently *Fasciola* parasites are thought to be able to not only evade host immune responses but also actively modulate immune responses (276; 272; 98; 270).

Fasciola immune evasion and modulation

Fasciola evade the host immune response in different ways. Newly excysted juvenile (NEJ) *Fasciola* penetrate the intestinal mucosa by secreting cathepsin-B and migrate into the peritoneum (197). At entry NEJs of *F. gigantica* produce stress responses, such as release glutathione S-transferases (GSTs) and anti-oxidant proteins, to inhibit local Th1 responses (197). *Fasciola hepatica* has been shown to secrete GSTs that block peritoneal macrophages from producing nitrites in rats (276). During migration the glycocalyx, a complex lipid layer secreted by the parasite, has been shown to protect *Fasciola* parasites from immune responses by changing surface antigens during migration (278). Subsequently eosinophils and antibodies fail to remain attached to the surface of the parasite during migratory phases (279; 280). Migratory *Fasciola* have also been shown to secrete ESP, such as cathepsin-L enzymes, during migration to provide protection by cleaving IgG and IgE from the tegument to limit ADCC and thus evading the immune response (281; 219; 282).

To promote their survival *Fasciola* infections appear to be able to evade Th2 responses and also down-regulate Th1 host immune responses in their favour (Figure 1.4.2). In early stage infections peripheral blood mononuclear cells (PBMCs) from *F. hepatica* infected cattle were shown to produce elevated levels of transforming growth factor B (TGF-B) and IL-10 (283). Once PBMCs production of TGF-B and IL-10 was blocked, IFN-γ responses increased indicating PBMCs down-regulate Th1 responses in early stages of *Fasciola* infection. Immune responses are down-regulated from the point when NEJ penetrate the intestinal mucosa to prevent enteric bacteria that may also enter triggering septicaemic responses (272). Secreted ESP molecules from *F. hepatica* infections during migration are likely to play a role in polarisation of Th2 responses in acute and chronic infections. Alternatively activated macrophages are stimulated from *F. hepatica* ESPs in mice and sheep with IL-10 production and

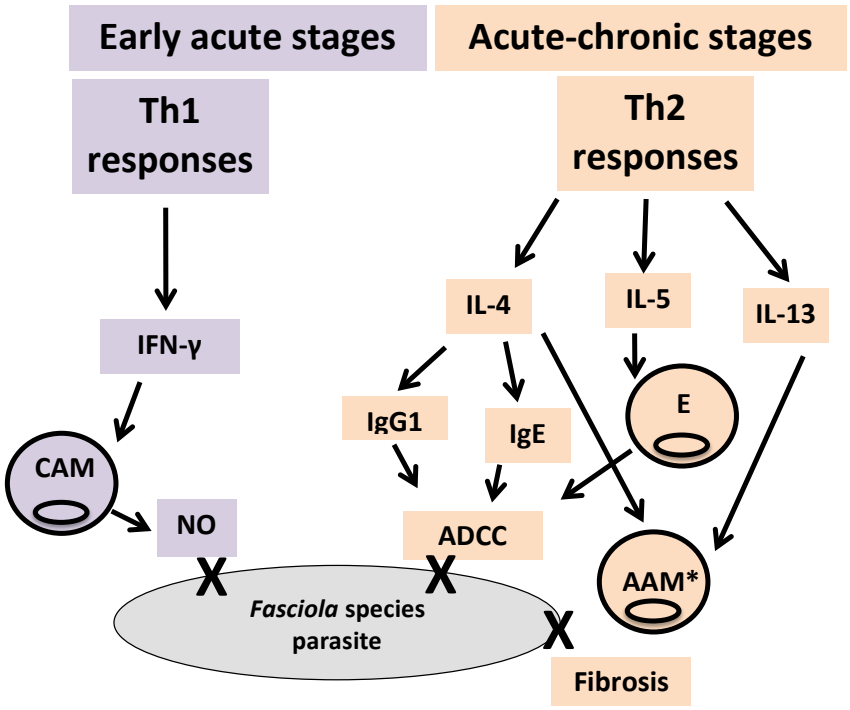


Figure 1.5: **A graphical representation of immunology of *Fasciola* species.** Adapted from (276). Purple: Early acute stages of infection; Orange: Acute-chronic stages of infection; *: Only demonstrated in *F. hepatica* infections; E: Eosinophil; CAM: Classically activated macrophage; AAM" Alternatively activated macrophage; ADCC: Antibody dependent cell cytotoxicity.

low levels of IL-12 (275; 284). Production of IL-4, IL-5 and IgG1 from stimulation of *F. hepatica* ESP in mice highlights Th2 polarisation (272). Furthermore chronic *F. hepatica* and *F. gigantica* infections demonstrate elevated IgG1 responses, with no IgG2 responses, highlighting polarisation of Th2 responses with absence of Th1 responses (285; 198). In a study by O'Neill and others, knockout mice were used to demonstrate the effect of *F. hepatica* burden upon immune modulation (286). Five different mouse strains, including a wild-type and IL-4 knock out strain, were infected with 5 or 15 metacercariae. All five strains had down-regulated Th1 responses and up-regulated Th2 responses, including the IL-4 knockout strain, with extreme Th2 polarisation with the higher metacercariae dose.

Few studies have compared the molecular biology, gene and protein expression between *F. gigantica* and *F. hepatica*. It is unlikely immune evasion and modulation strategies that occur are identical for *F. hepatica* and *F. gigantica* infections due to their genotypic and phenotypic differences. Excretory/ secretory, tegument proteins and GSTs are reported to differ between the two species and might be important in inducing species specific immune responses to infection (197; 282). Also the degree of immune evasion and modulation may also vary between *Fasciola* species (287). *Fasciola gigantica* infections in sheep, compared to *F. hepatica* infections, produce lower levels of cathepsin-B and subsequently *F. gigantica* more susceptible to elimination (282). Furthermore there maybe host differences in response to different *Fasciola* species. A breed of sheep, the Indonesian thin-tail (ITT) sheep, has been shown to be resistant to *F. gigantica* infections however remain susceptible to *F. hepatica* (288). Resistance is thought to be related to retention of Th1 responses (288; 287). Other sheep breeds, such as the Merino, do not retain these Th1 responses and remain susceptible to both *F. gigantica* and *F. hepatica* (289). Resistance to *F. gigantica* is thought to be related to the genotype of ITT sheep (270) but could also be down to the differences between *Fasciola* species phenotype.It is unknown if immune

modulation strategies of *F. gigantica* are the same as *F. hepatica* infections in cattle.

1.4.3 Bovine tuberculosis and *Fasciola* co-infection interaction

Co-infection dynamics

In natural populations, of animals or humans, multi-pathogen infections are common, compared to single infections, due to pathogen ubiquity within ecosystems (290). The terms "co-infection", "mixed infection" and "concomitant infection" have been used in infection biology to describe the presence of multiple pathogen infections that belong to a different strain, species or even phylum (291). Their interactions with the host and with each of the other pathogens is of interest as it maybe beneficial, detrimental to host and other pathogens or neither(292). As such the presence of other pathogens might influence development of disease as well as those immune responses used for diagnosis (293). For example modulation of host immune responses by helminth co-infections can result in altered immune effects to primary bacterial pathogens when present as multiple infections (98). Subsequently it is becoming increasingly clear that when investigating performance of diagnostic tests and describing epidemiology of a disease it is important take into account the presence of co-infections that might interact with the pathogen or disease of interest.

Helminth immune modulation characteristics have probably evolved due to their large nature and their multiple species/ environment life cycle stages; making it difficult for them to evade the immune response entirely (294). The degree and dynamics of the interaction between the host, co-infection and primary pathogen is important on the outcome of disease. For example previous exposure to *M. bovis* in humans was noted to have protection against *M. tuberculosis* in the early 20th century; a basis for

subsequent worldwide use of the BCG vaccine (67). However vaccine efficacy maybe affected by co-infections with helminths and vaccine failures may be due to active immune modulation by helminth species to aid survival within the host. For example reduced PPD intradermal skin responses have been reported in children co-infected with gastrointestinal helminths despite being BCG vaccinated to protect against TB (82; 294). Co-infection interactions have also been shown to affect host susceptibility to disease particularly with trematode co-infections (295). For example *M. tuberculosis* infected mice co-infected with *Schistosoma mansonii* have been shown to have down-regulated Th1 responses with increased Th2 responses (296). Co-infected mice had increased bacterial load and TB lung pathology compared to single *M. tuberculosis* infections.

Modulation of host immune responses by one infection may also impact on the immune responses normally produced by a primary infection. Such co-infection interactions potentially impact on the immunity, susceptibility, pathogenesis, clinical presentation, diagnosis and epidemiology of the primary pathogen within the host population (290). *Mycobacterium bovis* and *Fasciola* species as mono-infections cause specific immune responses and may progress to disease in cattle. In most cattle populations in SSA, there are few control programs against *M. bovis* infection. Subsequently *M. bovis* posing a potential risk to animal and human health, and diagnosis in individual cattle is important for surveillance and control purposes. *Fasciola* species are particularly common in SSA cattle populations globally and *F. hepatica* infections have been shown to modulate host immune responses although less is known about *F. gigantica* infections (276; 272). *Fasciola* co-infections appear to down-regulate Th1 immune responses within the host that are usually induced by other primary pathogens. The impact of *F. hepatica* co-infection has been studied in a range of primary infections including *M. bovis* although much less is known about *F.*

gigantica co-infections.

Immunity

In isolation *Bordetella pertussis* vaccination in mice produces Th1 responses demonstrated by production of IFN-γ (297). *Bordetella pertussis* vaccinated mice that were co-infected with *F. hepatica* metacercariae demonstrated marked Th2 responses such as production of IL-4 with absence of IFN-γ. Similar responses were noted if *F. hepatica* was administered pre- or post-vaccination. However these responses could be related to host response to *F. hepatica* rather than active immunomodulation by the parasite. In an additional part of the same study, knock out mice were also used to demonstrate the down-regulation of Th1 responses in favour of Th2 responses with *F. hepatica* co-infection (297). *Bordetella pertussis* vaccinated wild type and IL-4 knock out mice had dominant Th1 responses, demonstrated by IFN-γ production. *Bordetella pertussis* vaccinated *Fasciola hepatica* co-infected wild type and IL-4 knock out mice had down-regulated Th1 and dominant Th2 responses. Demonstrating that the effect of *F. hepatica* co-infection has influence of Th1 responses in absence of endogenous IL-4. Similarly cattle studies, infected with *M. bovis* and *F. hepatica*, Th1 responses down-regulated compared to *M. bovis* only infections. Specifically IFN-γ was lower in co-infected cattle, demonstrating a down-regulation in Th1 responses. Cytokine TGF-B and IL-4 responses were also elevated in co-infected cattle highlighting an up-regulation in Th2 response (298; 299).

Susceptibility

Infections with *F. hepatica* have been shown to increase the susceptibility of Th1-stimulating pathogens such as *Salmonella dubin*. *Salmonella dublin* infected

cattle, co-infected with *F. hepatica*, continued to shed *S. dublin* in their faeces for longer than *S. dublin* mono-infections (300). Brady and others demonstrated that *Bordetella* bacterial loads were were 40x higher in *B. pertussis* vaccinated mice co-infected with *F. hepatica* than those only vaccinated (297). In contrast cattle infected experimentally with *M. bovis*, *M. bovis* load was investigated with and without the presence of *F. hepatica* co-infection(301). Co-infected cattle had reduced IFN-γesponses and mycobacterial load within TB lesions. Similar findings were found with lower *M. tuberculosis* burdens in mice co-infected with the trematode *Schistosoma mansoni* (296). Highlighting that although *F. hepatica* co-infection reduces immune responses to Th1 producing pathogens, the net effect might increase or decrease the susceptibility to specific pathogens.

Pathogenesis and clinical presentation

The effect of *F. hepatica* co-infection on development of TB pathology has been investigated with *M. bovis* infection studies in cattle. Fewer TB lesions were noted in co-infected cattle and TB lesions had lower severity scores compared to TB lesions in *M. bovis* mono-infected cattle (298). Lower IFN-γ responses were also noted in co-infected cattle and potentially differences in bTB pathology were associated with down-regulated Th1 responses. Garza and others also demonstrated that cattle co-infected with *M. bovis* and *F. hepatica*, had lower IFN-γ responses with a reduced number of tissues infected. Although there was no difference in number or severity of lesions between *M. bovis* and *M. bovis* and *F. hepatica* co-infected cattle (301). Suggesting that influence of *F. hepatica* co-infection on development of bTB pathology maybe more complex than first thought.

Diagnosis

As highlighted, down-regulated IFN-γ responses were associated with *F. hepatica* co-infection. IFN-γ and other CMI responses are important in identifying bTB positive cattle and their down-regulation, with *F. hepatica* co-infections, leading to false negative bTB test results. A study by Flynn and others investigated BCG-vaccinated cattle and their responses to the SCITT and IFN-γ assay with and without *F. hepatica* co-infection (302). As cattle vaccinated with BCG are indistinguishable from *M. bovis* infected cattle using these diagnostics (78) vaccinated cattle were used as a model for *M. bovis* infection. Of the cattle vaccinated with BCG and co-infected with *F. hepatica* in the study, 8/9 tested bTB negative using the IFN-γ and 7/10 tested bTB negative using the SCITT. This highlights that *Fasciola* co-infection potentially can contribute to bTB false negative results. Claridge and others investigated SCITT responses in calves experimentally infected with *M. bovis* with and without *F. hepatica* co-infection (299). Although SCITT responses were reduced in co-infected calves all animals were still classified as bTB positive. This result suggests that *F. hepatica* co-infection down-regulates Th1 immune responses used for bbTB diagnosis but other factors might influence the magnitude of the down-regulation.

Epidemiology

One of the first co-infection associations noted was cattle being diagnosed as infected with *S. Dublin*, from clinical presentations of salmonellosis, often being co-infected with *F. hepatica* (303). Similar results were noted in a Dutch epidemiological study in dairy cattle, where herds with positive for *S. Dublin* were also associated with being positive for *F. hepatica* (304). Demonstrating that the relationship between the two infections has implications for infection control both at individual and herd level.

Determining herd status is important for the UK national test and slaughter control program towards elimination of bTB (305). In a recent study of English and Welsh dairy herds was conducted to understand the influence of *F. hepatica* on the epidemiology of bTB (299). A strong negative association between bTB prevalence and *F. hepatica* prevalence at herd level, for example where *F. hepatica* herd seroprevalence was high *M. bovis* herd positivity was low and vice versa. Furthermore *F. hepatica* co-infection was shown to decrease the likelihood of bTB being detected on individual farms using multivariate analysis. Such work displays the influence of *Fasciola* co-infection on bTB diagnosis in the UK cattle population, independent of other bTB risk factors, and the potential negative affects on the national bTB control programs. Similar results are postulated from Irish herds, where *F. hepatica* is endemic throughout the majority of the national herd. Studies conducted in various countries highlights the potential wider impact of *F. hepatica* co-infection, on bTB diagnosis and control, in cattle populations where *F. hepatica* co-infections are common (306).

Fasciola gigantica co-infection

The impact of *F. gigantica* co-infections have been minimally studied in any species. Reports of *F. gigantica* co-infection with other parasitises are frequent in sub-Saharan African cattle (307; 215; 308). It is unclear if *M. bovis* and *F. gigantica* co-infections will have similar outcomes in cattle to those identified with *F. hepatica* co-infections. One study in Zambia reports that *Fasciola gigantica* pathology was associated with presence of bTB lesions in slaughtered cattle using univariate analysis (309). Although only univariate analysis was conducted, the result indicates that co-infection outcomes may not be identical between to that of *F. hepatica* species.

Chapter 2

Cameroon and cattle

2.1 Introduction

Cameroon's geography and people have shaped the current state of cattle industry for centuries, which will have an impact on the epidemiology of bTB. Transmission of *M. bovis* is influenced by husbandry practices, interaction between cattle and other susceptible species (310; 26). Risk of zoonotic transmission of *M. bovis* will be affected by people's interaction with cattle, processing and consumption of meat and dairy products (22; 28; 25). In order to investigate the epidemiology of bTB in Cameroon it is important to understand the composition of the population cattle population within the context of the cattle industry. The composition of this cattle population, industry along with the data available and infrastructure present have influenced the design of studies in chapter 3 to answer the research questions outlined in the aims of this thesis (Chapter 1).

2.2 Geography and climate

Cameroon is composed of diverse terrains, situated within the transition zone between Central and West Africa below the Sahara desert. The south of the country is comprised of coastal beaches and mangroves that merge into extensive forest lowlands of the east, extending into the rocky mountains of the west. As the western mountains extend northwards the forests dissipate into fertile plains and into the savannah of the arid north of the country. Cameroon is bordered by Equatorial Guinea, Gabon and Congo to the south, the Central African Republic (CAR) and Chad to the east and north. Nigeria borders the entire west. The country is split into 10 administrative Regions, being further split into Divisions and Sub-Divisions (Figure 2.1).

The two regions of interest, in regard to this thesis, are the North West Region (NWR)

and Vina Division (VD) of the Adamawa Region (Figure 2.1). They are of relatively similar land area of 17,300km^2 and 17,196km^2 respectively. However the two study sites differ in both a physical and cultural sense. The NWR lies between 5.5^o and 7.5^{o}N and 9.5^o and 11.5^{o}W. It is characteristically mountainous with elevations of 700-3000 meters above sea level (mamsl). The terrain of the NW varies from rocky mountains to subtropical forests to fertile plateaux savannah. Smaller zones of fertile farmland and grassland savannah lead into the West and Adamawa Regions. Small rivers run throughout the Region, many swelling in the seasonal rains, along with smaller lakes dotted throughout the region including crater lakes such as Lake Nyos in the North and a larger plateau lake, Lake Bamendjing, in the South. In contrast the VD has a mountainous western boarder that turns into undulating grassland savannah eastward. Being 500-2500mamsl above sea level, the VD is situated between 6.5^o and 8^{o}N and 12.5^o and 15^{o}W. The Vina River, and its smaller tributaries, intersect the VD south-westwardly into patchy swamp areas.

Cameroon's climate, like much of the African continent, is dominated by the dry and wet seasons. The convergence of hot southerly winds, from the Sahara dessert, and the jet stream from East Africa result in these two seasons. The dry season occurs from September-November until April-June. When the humid jet streams from the southwest Atlantic ocean move eastward, across Africa's central belt, the result is the "Monsoon" wet season (311; 312). The change in monthly rainfall across the country varies within the two seasons. In general the NWR receives a longer wet season than the VD. Differences in temperature can also be noted between the two regions. In Bamenda, the capital of the NWR, January temperatures range from 12^{o}C to 19^{o}C in January and 6^{o}C to 18^{o}C in July. In contrast, Ngaoundere, the capital of the VD and Adamawa Region, experiences higher temperatures due to its generally lower elevation. Temperatures in January range from 22^{o}C to 24^{o}C and in July from 18^{o}C to

20^{o}C. Bovine parasitic infections require specific environmental and climatic conditions for completion of their life cycles. Hence differences in climate in the NW and VD may affect incidence of bovine parasitic infections such as *Fasciola* species. Although minimally investigated in Cameroon seasonal and region variation in incidence of bovine *Fasciola* infections has been identified in other countries with wet and dry seasons (313).

Cameroon's varied geography and climate influences national cattle production practices with the majority of cattle production taking place in the NWR, Adamawa, North and Extreme North Regions (Figure 2.1). In both study sites the grazing pasture is "savannah" type including *Hyparrhenia* and *Sporobolus* grasses with sparse tree cover (314; 42; 315). Private fenced pasture, where it exists, often includes *Brachiaria* grass species introduced to improve grazing quality. Frequency of rain influences availability of pasture and subsequently cattle grazing. Some herds move to communal pasture in the dry season when pasture is scarce termed transhumance. Wet season rains also have influence on where cattle are taken to drink with many smaller streams and rivers vanishing in the dry season. Transhumance patterns can influence infectious disease transmission in livestock (316; 317). Lightening storms can cause sporadic cattle mortality due to direct lightening strikes.

2.3 Human population

Cameroon's population currently stands at 23,924,000 and is mainly centred around regional capitals especially in the Central and Littoral regions (318). Francophones make up approximately 4/5 of the population with anglophones being the remainder (312). The country is still linguistically diverse with 250 ethno-liguistic groups within the country in addition to the official languages of French and English (319). Of

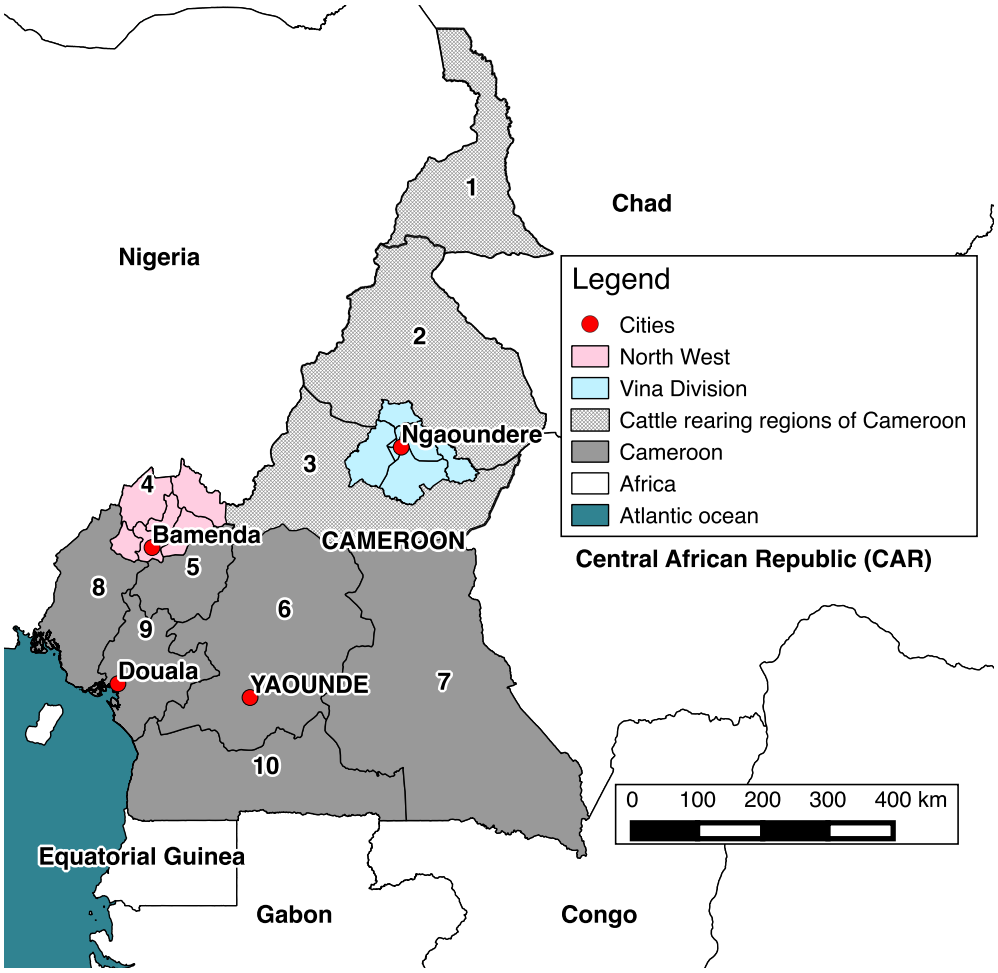


Figure 2.1: **Map of Cameroon and adjacent countries.**
The 10 administrative Regions of Cameroon are numbered (1= Extreme North, 2= North, 3= Adamawa The Vina Division within the Region is highlighted. (VD = light blue)), 4= North West (NWR = pink), 5= West, 6= Central, 7= East, 8= South West, 9= Littoral and 10= South). Cities of interest are highlighted by red points. Cattle producing areas are highlighted in light grey, light blue and pink. The highlighted VD (Light blue) and NWR (Pink) are the study areas investigated in this thesis.

particular importance, to the cattle industry, is the Fulani language of Fulfulde. The language demonstrates the importance of cattle to the Fulani, with having 74 words for "cow"(320), with this group keeping cattle as part of their culture since the 13th century (321). Other ethnic groups, who keep cattle, use Fulfulde for the purposes of cattle trade.

The population of the NWR is 1,804,695 predominantly based around the Region's capital city Bamenda and other towns around located on the ring road within the Region (319). The NWR is predominated by Bantu speaking groups, such as Mboum and Bali, with some cultural similarities to Nigerian Bantu ethnic groups. Aside from the formal regional government parts of the region are still organised into local grassland chiefdoms. Annual festivities take place at in the chiefdom palace celebrating local traditional beliefs. Christianity is the predominant religion, after traditional beliefs, with the muslim faith being the majority of the remainder. The muslim faith is practiced by the Fulani and Hausa groups.

The VD has a much smaller population size, than the NWR, of 317,888 individuals. The majority of people are based around the city of Ngaoundere (152,698)(319) and the remainder are dispersed in villages and a few small towns. The majority of the VD population is of muslim faith and Fulani ethnicity. Smaller christian Bantu communities, such as Mboum and Baya groups, exist mainly around Ngaoundere. Local Fulani chiefs or "Alahajis" govern a village and Ardo'en advise and represent local Fulani herdsmen. Alahajis and Ardo'en have local political influence at weekly events such as local cattle markets and at muslim festivities like the "Fete de Mouton". Fulani groups in the NWR have similar leaders although they often have less significance outside the Fulani communities. In both the VD and NWR ethnic, cultural and religious tolerance is high allowing integration of different groups within the same geographical area.

2.4 National governance and infrastructure

The Republic of Cameroon's government is based in the capital of Yaounde. Each of the 10 administrative regions in Cameroon is governed locally by a regional government. The North West's regional capital is Bamenda and Cameroon's third biggest city. The city of Ngaoundere, within the VD, is the capital of the Adamawa Region (Figure 2.1). Regional capitals house regional government offices for example in education, health and veterinary sectors. Both Bamenda and Ngaoundere have their own universities with additional universities in other major cities such as Yaounde. Local primary and secondary schools are either private or state run mainly requiring fees for attendance and often faith based. Medical facilities are centralised in cities and towns including state-run, religious and private hospitals with varying facilities. Many medical facilities are supported by international non-governmental organisations (NGOs). Rural communities often have to travel to the nearest urban centre for medical care as local clinics often have minimal facilities or simply do not exist. The national tuberculosis program is heavily funded by international organisations, predominantly American, French and British, such as the Global Fund and World Health Organisation (WHO). The program is managed on a Regional basis with distribution of this funding varying between and within Regions. Tuberculosis diagnostic and treatment centres are present within all Divisions in the NWR with varying facilities. Conversely TB medical centres within the VD are centred within Ngaoundere and access maybe be limited in rural communities (322).

The national transport network is centred around Yaounde and the industrial capital of Douala. The distribution of the transport network is related to business, import and export of goods both nationally and internationally. Yaounde and Douala have international airports. Roads stemming from these two centres are, in general, sealed

leading to other Regional centres. Heavy persistent rainfall in the wet season can disrupt road transport as most roads are not sealed. This creates suspended or dangerous travel, by vehicle, especially in the mountainous NWR. Cattle are also historically transported by road or cross-country for trade purposes usually on foot. Goods and livestock are often transported on these roads by lorries, despite the weather conditions, which adds to road erosion. Transport on some roads has a risk of highway robbery for people travelling in isolated areas as police presence is mainly restricted to towns. Private bus companies transport passengers and post between and within regions. Communal taxis and motorbikes, called okatas or motos, transport individuals at a local level. In the NWR a ring road runs from Bamenda circumferentially around the region, varying in road quality, and back through many of its divisional centres. This connects the region to Bamenda and on to Yaounde eastwards. But the region itself is fairly enclaved from the rest of Cameroon. Minor routes exist for transport to other regions, such as to the Adamawa region, and to small border crossings to anglophone Nigeria. In the VD; Ngaoundere is connected to Yaounde in the south and to northern regions by a well maintained sealed road. Facilitating transport of goods within Cameroon and onwards to adjacent countries. A railway also exists between Ngaoundere and Yaounde mainly transporting passengers, livestock and post. Communities situated in rural areas away from the main road and rail networks are subsequently more isolated than populations in urban centres. Communication has been improved with the advent of mobile phone networks in the past 10 years but signal is variable. Internet access is restricted to regional capitals and a few divisional towns.

Piped water is available in cities and towns; through community taps or in some cases by direct pipes to houses. In rural communities water is supplied by bore hole hand pumps. Nomadic communities also collect water directly from natural water sources

and may also share these water sources with livestock. This is a notable point as sharing water sources may be a point of *M. bovis* transmission between livestock (323; 324) and humans (325; 326). The national energy network supplies regional capitals and towns mainly being produced by hydroelectric systems or large carbon fuel generators. Often smaller remote communities, even within a few kilometres of an urban centre, will not be supplied. Frequent power-cuts are noted, particularly in the NWR for hours to days, during peak energy use at night or in the wet season where weather conditions can disrupt power supply. Energy disruption can cause problems where a cold chain is required such as with vaccine distribution and food refrigeration. As a consequence livestock are transported live and slaughtered at the point of consumption. Smaller communities and vital services such as hospitals and laboratories often rely on smaller petrol powered generators for backup electricity supplies.

2.5 Veterinary sector infrastructure

Ministry of Livestock, Fisheries and Industrial Agriculture

Livestock production is under the responsibility of the Ministry of Livestock, Fisheries and Industrial Agriculture (MINEPIA). The ministry is arranged in a hierarchical structure, the MINEPIA Minister based in Yaounde, a head of veterinary services and a delegate are based in each regional capital. Within a Region each Division has its own delegate, subdivisional delegates and local chiefs of centre sometimes with veterinary support staff. Each position, in theory, is based in the vicinity of the area of jurisdiction. However in many remoter centres local chiefs of centre and veterinary staff will base themselves in the nearest regional town. The central office for the NWR is situated in Bamenda and administers 7 Divisional, 35

Sub-Divisional and 81 local veterinary centres. The Adamawa, administered from Ngaoundere has 5 divisions; with the VD being one of those. The VD has 8 Sub-Divisional and 31 veterinary centres.

Veterinary services are delivered from local Veterinary Centres, or Centre Veterinaire et Zootechnique, (VC) by the chief of centre and veterinary support staff. Livestock species covered by each VC vary, depending upon area, but can include cattle, small ruminants, pigs, poultry, fish and companion animals. Chiefs of centre are usually veterinary technicians or occasionally qualified veterinarians. Veterinarians in general work in higher government, including administration and research, with a few in private veterinary companies or NGOs. Veterinary technicians are trained at agricultural technical colleges in Jakiri, NWR or in Maroua in the Extreme North Region (Figures 2.4 and 2.5). Technical subjects covered in such training include agriculture, animal health, basic veterinary skills and veterinary public health principles such as meat inspection. The University of Ngaoundere offers a bachelors degree in veterinary sciences and historically many Cameroonian veterinarians have been trained in Nigeria and Senegal. VCs vary in their size but commonly centres cover 50-300 herds of cattle shown in appendix (Appendix A). Individual VC responsibilities do vary but can include:

- Transfer advice and policies from government direct to herdsmen.
- Record livestock and herdsman data within their locality.
- Coordinate and conduct annual vaccination campaigns.
- Regulation of local livestock markets.
- Regulation of municipal regional abattoirs and licensed slaughter slabs.
- Veterinary clinical work.

Physical facilities at the VC vary from a basic structure with no utilities to a more substantial building with electricity for vaccine refrigeration and a motorbike for transport. Cattle handling kralls are often based away from the centre for annual vaccination campaigns. Presently annual vaccination campaigns offer voluntary cattle vaccination for lumpy skin disease, clostridia, contagious bovine pleuropneumonia (CBPP) and anthrax. Trials of foot and Mouth (FMD) vaccination started in 2014. Veterinary centre records are commonly updated at annual cattle vaccination campaigns; recording by hand the number of cattle vaccinated by each herdsman or owner (Section 2.6). Separate vaccination campaigns occur for other species but no records are kept of the owners or numbers vaccinated.

Whether other clinical services are offered depends upon individual VC staff expertise. Diagnostic services at each centre are usually limited to clinical examination of animals. Diagnostic tests for bTB are not available at VCs and cattle suspected to be infected based are not recorded. Currently there are no regional or national laboratories conducting performing routine veterinary diagnostic services in Cameroon. Reporting of infectious diseases present in cattle at a VC or within its jurisdiction are based on presence of clinical signs. For example herd outbreaks of FMD are reported by VC to Regional offices as clinical signs are relatively specific for FMD and is regarded as a disease of national importance.

Additionally MINEPIA veterinary technicians undertake the meat inspection service at abattoirs and slaughter slabs. Only gross diagnoses are made as laboratory diagnostics are not routinely available. Meat inspection primarily focuses upon detecting zoonotic disease. For example meat inspection is the only routine form of bTB surveillance in cattle populations in Cameroon. Meat inspection also concerns infections which affect meat quality such as *Fasciola* infections of the liver. Basic records are kept but inspection quality varies between individuals and abattoirs

(Section 2.6).

MINEPIA also has a department for improvement of cattle production; The Livestock Development and Husbandry Corporation (SODEPA). Its aim is to improve cattle breeding, husbandry and production economics to herdsmen and butchers. In particular, breeding improvement is focused on Gudali, Simmental and Holstein cattle breeds. SODEPA has four large cattle breeding ranches including two in the NWR and one in the Adamawa. SODEPA also manages the countries two largest abattoirs in Yaounde and Douala. Recent work includes education of herdsmen on development of communal pasture and improving their access to artificial insemination (AI) services. A small number of private dairy and beef ranches also exist offering private AI services.

A growing number of private veterinary services exist in urban centres. They are mainly concerned with sale of pharmaceuticals to pig and poultry industries or companion animal health in Yaounde and Douala.

The Ministry of Scientific and Technical Research

The Ministry of Scientific and Technical Research (MINREST) has a division responsible for veterinary research entitled the Institute of Agricultural Research for Development (IRAD). There are four main IRAD research centres including Bambili near Bamenda in the NWR and Wakwa near Ngaoundere, Adamawa region. Both have scientific facilities and livestock for research purposes. Over the past 10-20 years facilities have become underfunded, with poorly maintained facilities and subsequently undertake fewer research projects. Production of livestock vaccines is undertaken at a facility near Garoua, North region, that is well resourced or vaccines are imported from Uganda and South Africa.

2.6 National agriculture and livestock production

Agriculture accounts for 53.3% of national employment (318). Much of the western and southern regions are still dedicated to crop production in particular palm oil, rubber, tuber crops such as cassava, cacao, coffee and tropical fruits. Large plantations dominate the South West Region producing palm oil, banana and rubber for export. Many of the crops produced for national consumption are from the NWR. Since 2005 there has been a shift in overall trade of products towards imports of $5.1 billion in value. Despite this trend, agricultural production contributes largely to the export offset. For example in 2011, 72.9% of Cameroonian exports were bio-oil products, crops and livestock; amounting to $2.1 billion of export value (327).

As highlighted previously cattle production is an important part of Cameroon's livestock industry both at a local and national level. Since the year 2000 the estimated size of the cattle population has increased from the estimated 4,976,000 to 6,040,000 in 2010 (319). Cameroon is also an important livestock producer within the Central-West African region with cattle moving across boarders between Chad, Nigeria and the CAR so the cattle population is likely to have increased since the 2010 records. Additionally there is significant importation of dairy products where there is a domestic shortfall hence it is not surprising that there is an drive for increased efficiency of meat and milk production within Cameroon for both national and international resale. The value of the cattle industry in Cameroon is currently unquantified.

In addition to cattle, other livestock industries are of national importance including pig and poultry production. Intensive pig and poultry industries are mainly centred within southern urban centres separate from muslim communities such as the West and Littoral Regions. Sheep are kept extensively with cattle in pastoral communities.

Goats, as well as pigs and poultry, are kept at a rural smaller holder level for personal consumption and microfinance. Promotion of small scale fisheries is increasing in recent years due to increase reliance on imports.

Pastoral and dairy cattle populations

The extensive distribution of cattle across the country makes estimating the absolute size of the cattle population in Cameroon logistically difficult. Furthermore culturally it is insensitive to ask a Fulani herdsman how many cattle they keep as the number of cattle owned is regarded as representative of an individuals wealth (314). Statistics on the cattle population size are published by the Cameroon Institute of National Statistics in Cameroon based upon the numbers of cattle for cattle vaccinated at VCs, recorded as slaughtered at abattoirs and traded in markets by MINEPIA staff. Since the year 2000 the estimated size of the cattle population has increased from the estimated 4,976,000 to 6,040,000 in 2010 (319). Although the institute does not publish statistics for specific Regions or Divisions in Cameroon these can be estimated from cattle vaccination records held in Regional MINEPIA offices.
The 10 regional MINEPIA offices hold VC cattle vaccination records and can be estimate the number of cattle herds by Region, Division or VC. Information collected at annual vaccination programs include the total number of cattle presented, cattle keepers name and their location. By visiting individual VCs across the NWR and VD the cattle population the most recent vaccination records can be collated (Appendix A). From the 2013 vaccination records 546,508 cattle from 5,053 herds were registered in the NWR and 176,257 from 1,927 herds in the VD. Cattle herds within the NWR and VD are distributed within areas of greater grazing area, such as Bui and Donga-Mantung in the NWR, and fewer in urban areas such as Ngaoundere II in the VD (Figure 2.3). The majority of herds range from 50-150 cattle throughout the

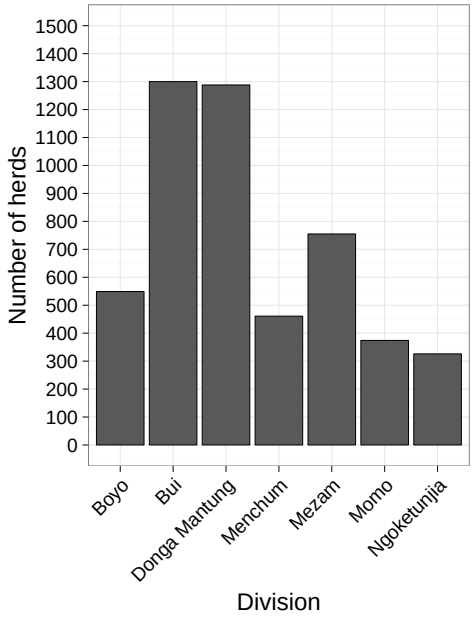
NWR and VD (Figure 2.3). However there are various reasons why using vaccination records are likely to underestimate the size of the cattle population. Recording precise numbers of cattle vaccinated is difficult as taxes are collected per animal presented. Although cattle keepers need to be registered to trade cattle, many avoid vaccinating all cattle to minimise taxation costs and avoidance is anecdotally high in Cameroon (328; 314). Although regarded as few in number cattle keepers who do not trade or vaccinate cattle will not be included in these records. Also pastoral cattle rearing involves nomadic grazing and may lead to not all cattle being present at the time of vaccination. Furthermore herds maybe registered at more than one VC if cattle graze over large areas. One owner may sub-divide his herd to minimise impact from outbreaks of infectious diseases such as FMD. Additionally regional, national and international boarders in Cameroon are permeable to unregulated cattle movements (315; 120; 329) and facilitates not all cattle being registered in some herds. These factors should be taken into account when interpreting population estimates from vaccination records.

The pastoral Fulani dominate Cameroonian cattle production as they do in many other West African countries (330). Traditional "Fulani" breed cattle are kept by the Fulani. Pastoral cattle are *Bos indicus* species with characteristically long horns and favoured for their resilience during nomadic grazing (331). Aku and Jafun subgroups of the Mbororo culturally prefer to rear White and Red Fulani cattle respectively. Gudali cattle are also kept which per animal produce more meat than Fulani breeds which are predominantly kept in the VD (314). Other minor *Bos indicus* breeds exist in Cameroon such as the trypanotolerant Namshi in the Extreme North region. European imports of beef cattle and semen, as previously mentioned, are an attempt to improve productivity of cattle through cross breeding (332). The composition of the population or herd structure in the NWR and VD have been minimally described.

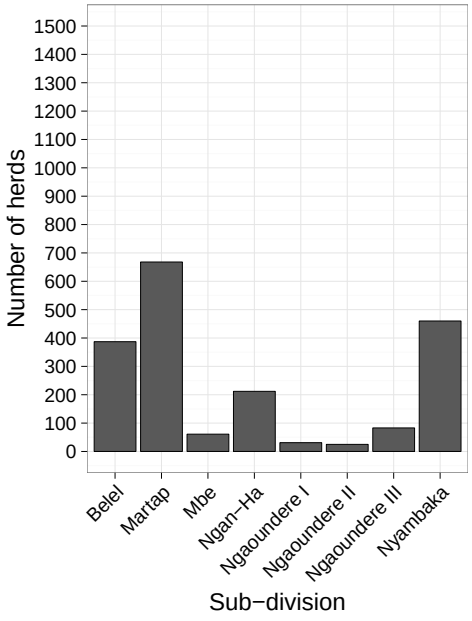
Previous studies in the VD report that female (48.8%) and male (51.2%) cattle are present as similar proportions with natural breeding accounted for 87.4% of all cattle breeding practices (314). However similar studies have not been conducted in the NWR to compare differences in composition of the cattle population. The majority of pastoral cattle are extensively grazed on communal pasture. In the wet season cattle are often grazed within the herdsman's local area. However during the dry season months, when availability of adequate pasture reduces, cattle are taken in search of pasture called "Transhumance". In the NWR fenced pasture is more common with increased land pressure from crop farmers. However intensive pasture management in Cameroon is in its infancy mainly on government owned ranches (333). During the dry season cattle, are also supplemented with salt and occasionally cotton seed cake for nutritional purposes. Access to water tends to be from natural sources dependant upon season. Fulani herdsmen traditionally co-graze sheep with their cattle and in some regions use horses for transport (314; 334).

Small dairy cooperatives exist in the NWR, often linked with NGOs, in peri-urban environments aiming to diversify individual small holder livelihoods established in the 1990s. Dairy cattle are reared independent of pastoral Fulani herds and is considered a separate industry. Hence dairy herd vaccination is recorded separately at MINEPIA NWR Regional office in Bamenda with similar records being kept to pastoral herds (Appendix B). Herds in the NWR are organised into 7 cooperative groups for milk processing and breeding purposes. From 2013 records 492 cattle were kept by 229 farmers with a range of 1-4 cattle being kept by each farmer. Hence dairy cattle make up a much smaller proportion of the cattle population in Cameroon compared with pastoral cattle. Cattle are managed semi-intensively in stall housing being fed cut-forage and occasional cotton-seed cake. There is little specific information about the composition of peri-urban dairy cattle but dairy cattle are

mainly *Bos taurus* dairy breeds such as Holstein-Frisians and Jerseys originally imported from Kenya or Ireland (335).

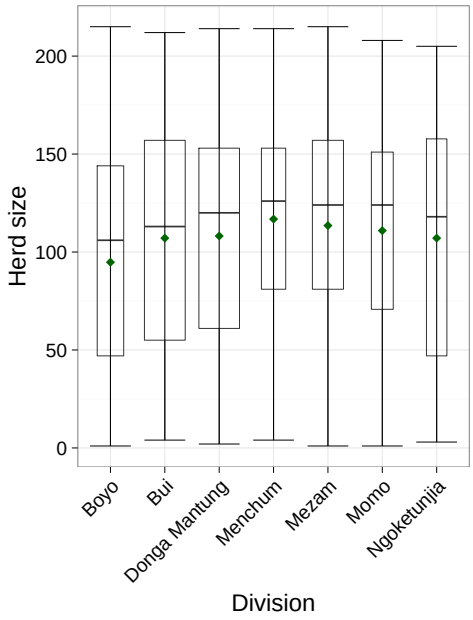


(a) North West Region by division.

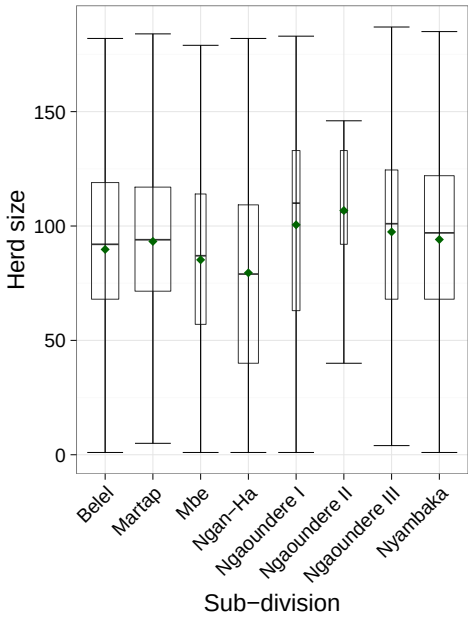


(b) Vina Division by sub-division.

Figure 2.2: **Number of pastoral herds registered for vaccination at veterinary centres in 2013.**



(a) North West Region by division.



(b) Vina Division by sub-division.

Figure 2.3: **Range in size of pastoral herds registered for vaccination at veterinary centres in 2013.**

Industries associated with cattle production

Local trade of cattle generally occurs in weekly markets and are often regarded as social events by the local Fulani community. Some markets may be nothing more than a gathering of herdsmen to physical structures with associated commodity markets. Unregulated sale of veterinary pharmaceuticals often occurs at such markets. Cattle tend to be bought from herdsmen by cattle dealers called "buyem and sellems" (42). Veterinary centre staff collect taxes, on behalf of MINEPIA, for cattle sold at markets although the composition of the cattle population traded has been minimally investigated. Buyem and sellems may transport cattle to further markets in regional towns for sale and possible slaughter. Figure 2.4 shows the suspected regional cattle movements in the NWR where movements appear relatively enclaved. In the VD cattle maybe traded from northern regions, Nigeria and Chad. Cattle may subsequently pass to Ngaoundere for further sale and to Yaounde, in the Centre Region, and Douala in the Littoral Region either by rail or road transporting many cattle for slaughter. Cattle are also transported from Bamenda to Yaounde, and to a lesser extent to Buea, by road or by foot. Suspected country-wide movements are summarised in figure 2.5.

Butchers purchase cattle directly from weekly cattle markets or buyem and sellems to be slaughtered (42). Reasons for resale of cattle by Fulani herdsmen include financial, for own consumption and due to sickness although this is anecdotal. Therefore the composition of the cattle population in the Region may not mirror the those entering the food chain in the abattoir. Slaughter occurs in smaller towns at slaughter slabs or regional government-regulated municipal abattoirs. For example abattoirs are present in regional capitals of Bamenda and Ngaoundere (Figure 2.4). Depending on their capacity abattoirs can slaughter as little as 10-35 animals per day in Bamenda at

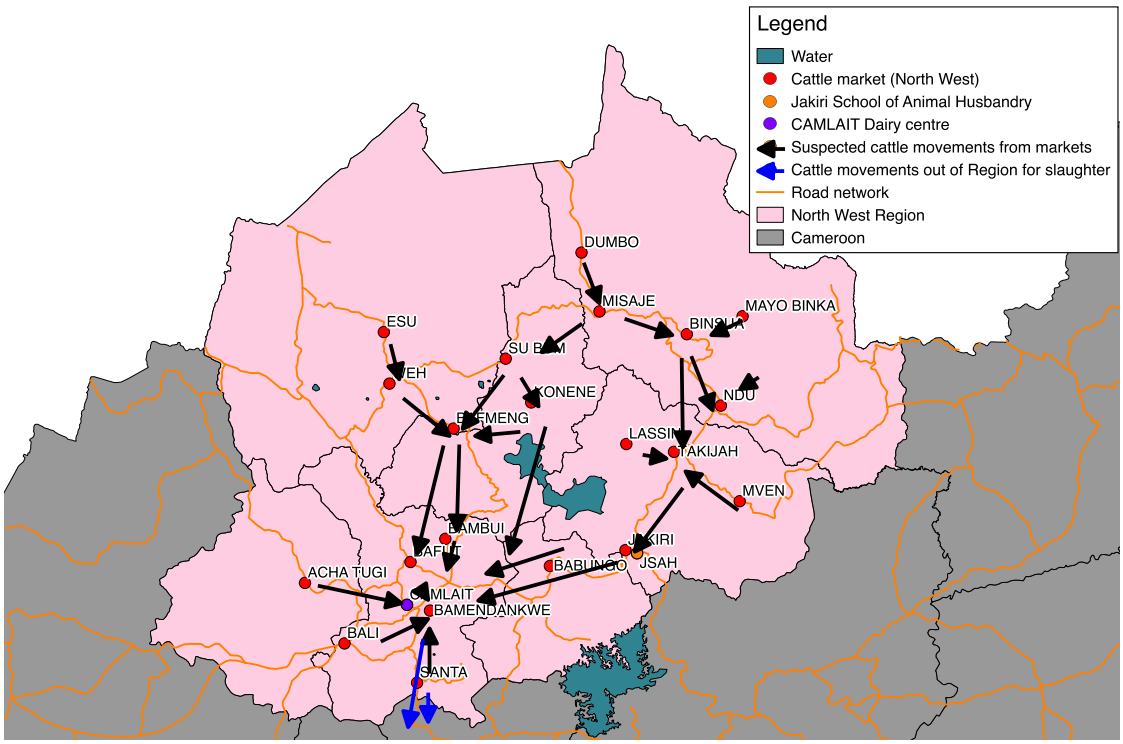
weekends and up to 400 animals per weekday in Yaounde. Mechanisation in abattoirs is absent or defunct. Cameroonian law dictates all slaughter must be performed by muslim slaughter men in a Halal manner. Independent butchers buy live animals to slaughter and employ staff to butcher meat by hand. Subsequently butchers pay taxes per animal for the slaughter service and meat inspection by MINEPIA veterinary technicians. A similar process is conducted at slaughter slabs with usually 0-2 cattle being slaughtered per day in towns. Anecdotally there is very little illegal slaughtered conducted in Cameroon.

As part of butcher tax collection for cattle slaughtered; MINEPIA veterinary technicians record information on paper about cattle slaughtered. Paper records at Douala and Yaounde Regional abattoirs date back since the 1990s (114; 75). Similar unpublished records are held in Bamenda and Ngaoundere in Regional MINEPIA offices. The type of cattle information collected by veterinary technicians varies between abattoirs but includes animal origin, sex, breed, age, whether part or all the carcass was condemned and the reason for any condemnations. Although prevalence of pathologies in cattle, such as bTB lesions and *Fasciola* infections (Chapter 1), have been previously published little is known about the composition of the cattle population slaughtered. Presently the only routine bTB surveillance of bTB in Cameroon is identification of TB lesions in abattoirs (Section 1.2.4) and described later in this thesis (Section 3.2.1). Other than partial or total carcass condemnation of the affected carcass it is unclear how these records are utilised. Parts of whole carcasses condemnations financially impact on butchers from the loss of product for re-sale. Anecdotally butchers followed up financial losses, due to condemnations associated with diseased cattle, from intermediaries and owners of the cattle although it is unclear how this is conducted in practice.

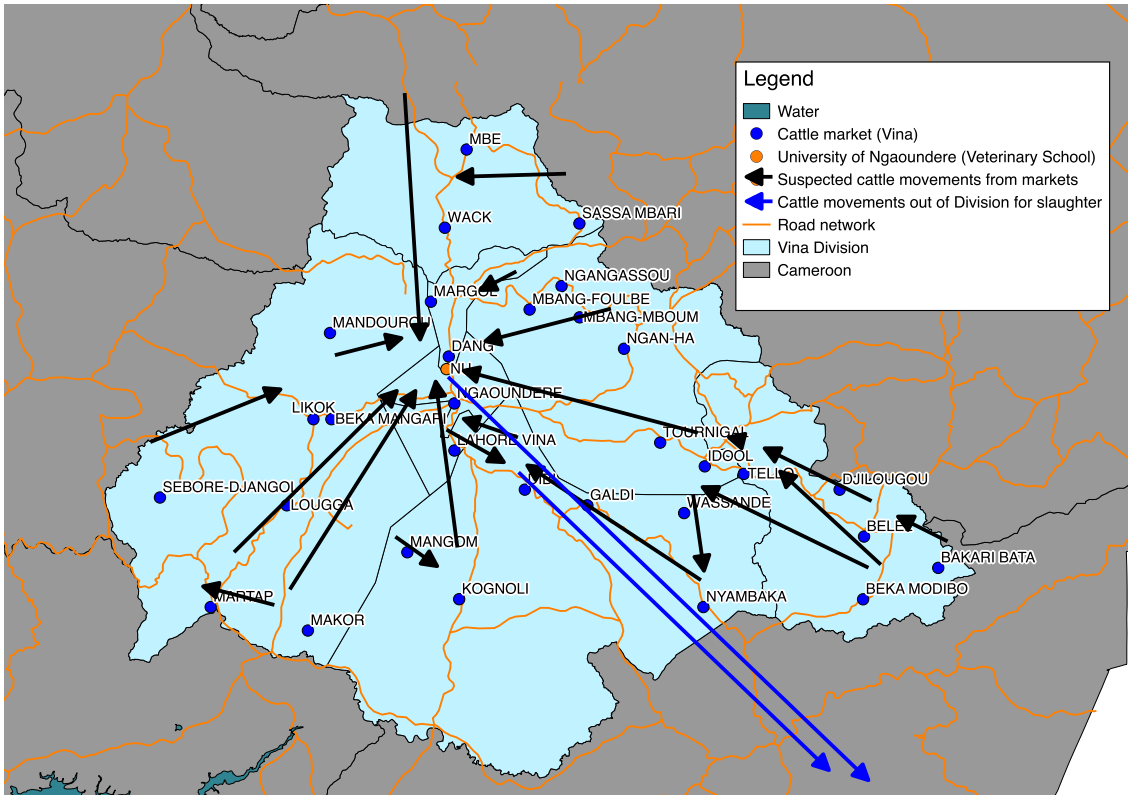
Meat is often sold outside the abattoir or transported to local commodity markets. It is

popular within markets and towns to consume fire-grilled beef or goat covered in spices called "Soya" accompanied by plantain or cassava. The Adamawa Fulani also consume a sun dried beef version, ideally preserved for long transhumance journeys, called "Kilichi" that can be kept for many months similar to biltong in South Africa. Other meat dishes tend to be in the form of slow cooked stews with spices varying in regional cultures.There are few abattoirs for slaughter of other livestock species and are generally poorly regulated.

Fresh milk is available within cattle rearing areas of Cameroon rather than nationwide (42). The majority of fresh milk is consumed by Fulani families at a local level. In Fulani culture milk is generally consumed on its own, mixed with spiced tea, "Chai", or as a porridge made from cassava. Individual Fulani families sometimes boil or sour milk prior to consumption. However the frequency of treatment prior to consumption is unknown. Other dairy products are also occasionally produced to preserve milk such as yoghurt and butter. Local resale of milk to non-cattle owning communities occurs at markets particularly within the Adamawa region. Other ethnic groups in cattle rearing communities consume milk to a lesser extent. In peri-urban dairy cooperative herds milk produced will be sold locally, as a cooperative venture, or processed into yoghurt, frozen milk (known as "Alaska" in Pidgin) or cheese. Larger dairy processing units exist, such as the government owned Camlait, but milk is often imported in powder form to produce dairy products such as yoghurt sold in cities. However public health monitoring of fresh milk is minimal if absent in rural regions of Cameroon.



(a) Suspected cattle movements from markets in the North West



(b) Suspected cattle movements from markets in the Vina

Figure 2.4: **The location of cattle markets, present in 2013, in the North West Region and Vina Division, Adamawa Region.**
Additionally the suspected movements of cattle between markets to slaughter at large regional abattoirs is also shown.

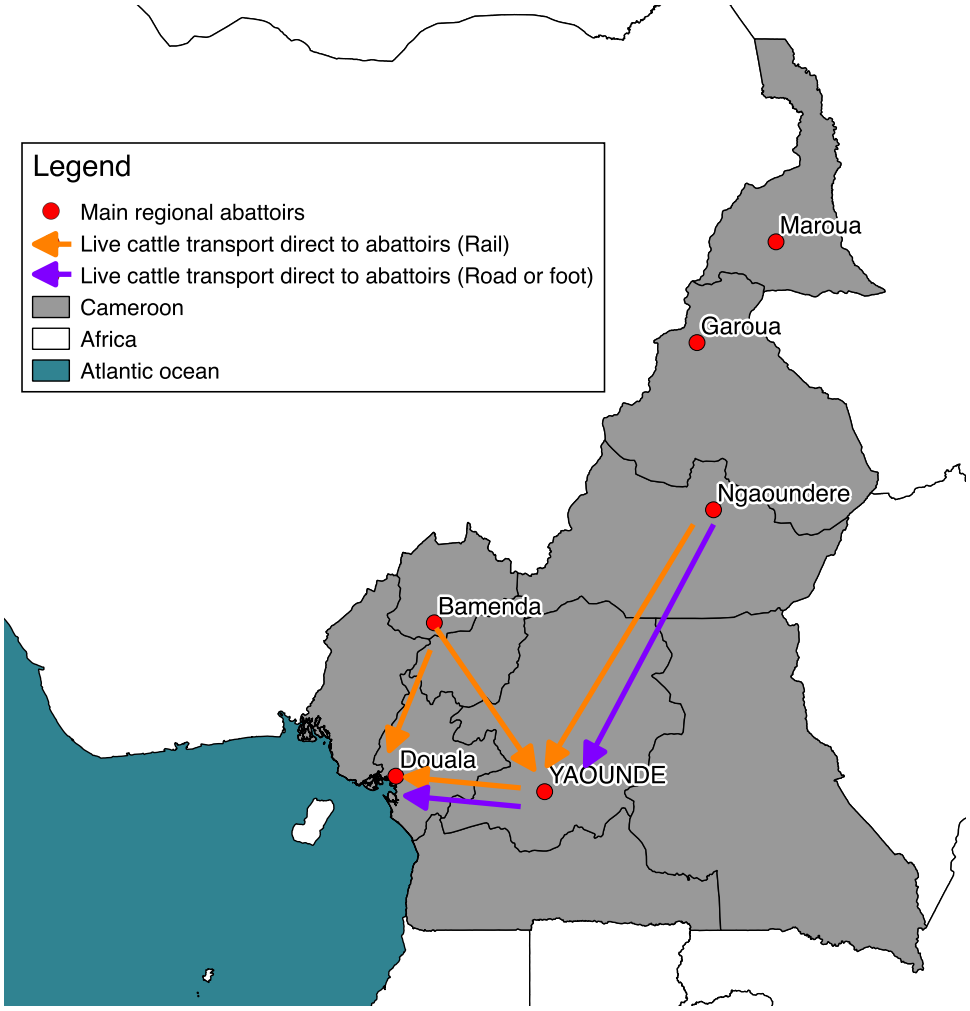


Figure 2.5: **The location of large regional abattoirs and major movements of cattle for slaughter between regions.**

2.7 Summary

In conclusion the shape of todays cattle industry in Cameroon has been influenced by local geography and interactions between different cattle rearing communities. Veterinary centre vaccination and abattoir slaughter records can be used to describe the current population of cattle in the NWR and VD. Although the variation in composition and husbandry practices between the two areas has been minimally investigated. The background of the cattle industry provides an understanding of the distribution of cattle, cattle husbandry practices and highlights the potential causes infectious disease transmission such as bTB.

Chapter 3

Materials and methods

3.1 Introduction

This thesis encompasses work from a larger bTB epidemiological project in Cameroon titled "The Epidemiology and Phylogenetics of Bovine Tuberculosis in Cameroon, Central Africa" (Chapter 1). This chapter covers describes the studies conducted as part of this thesis:

1. A convenience based abattoir study of slaughtered cattle in Bamenda (NWR) and Ngaoundere (VD).
2. A population based cross-sectional study of pastoral cattle herds based in the NWR and VD.
3. A smaller cross-sectional study of dairy herds in the NWR Region.

Including explanation of the study design, sampling, laboratory and major statistical methods conducted to answer the research questions outlined in Chapter 1.

3.2 Study designs and sampling

3.2.1 Abattoir study

Study sites

An abattoir study was conducted in two municipal abattoirs situated within the 2 study sites in the cross-sectional study (Figure 2.1). Abattoirs were the central municipal abattoir in Bamenda, NWR and the central municipal abattoir in Ngaundere, VD, Adamawa Region. The abattoirs were the only large scale abattoirs within the two regions other than smaller community slaughter slabs which may slaughter 0-3 cattle daily. Individual butchers bought cattle from local cattle markets

or intermediaries within the week prior slaughter. Both abattoirs slaughtered cattle daily, from about 5am for 1-2 hours per day, only slaughtering cattle. Bamenda slaughtered between 8-60 cattle and Ngaoundere between 20-60 cattle per day. Infrastructure at both abattoirs encompassed a pasture holding area for live cattle and a simple shed building for slaughter and meat inspection. Water and electricity supply was variable from day to day. A team of MINEPIA trained meat inspectors were present to register cattle, collected slaughter taxes from butchers and inspected meat prior resale. Butchers generally employed assistants, who may work for more than one butcher, to aid restraint and butchering of cattle. Once cattle were restrained within the slaughter house a designated muslim slaughterman would conduct the halal slaughter by using a single laceration of both jugular veins without stunning. Butchers would mark meat with unique laceration marks, using a butchers knife, to identify their own carcass. Meat inspectors then inspected the individual components of carcasses prior re-sale. The degree of meat inspection varied between and within the 2 abattoirs. However to particular attention the head, neck, pluck and liver for lesions suspicious of tuberculosis and fasciolosis. Routinely parts of organs, the carcass or all the carcass was condemned, depending upon the severity of pathology, and disposed of in closed refuse pits. In general all parts of the carcass were sold the same day either directly to customers outside the abattoir or transported to local food markets for resale (Figure 3.1).

Study design

Convenience samples were taken from the 2 abattoirs with the aim to sample all cattle presented. The primary aim of the study was to assess prevalence of bTB using a imperfect diagnostic test based on PME lesion identification (138). Based on previous estimates from the NWR we assumed a prevalence of lesions of $\sim$5% (75) and

calculated a target sample size of 1015 cattle per abattoir (Survey Toolbox; AusVet) (336). This would allow the within abattoir prevalence of 5% to be estimated with a precision of 5% with a 95% level of confidence. Hence the aim was to sample between 1000-2000 cattle per abattoir. Sampling took place in February-July 2012 in Bamenda and July-August 2013 in Ngaoundere over a consecutive period.

Sampling methodology

In this instance, a convenience sample was defined as sampling all the cattle presented for slaughter on each day within the study period for each abattoir. Each day cattle to be slaughtered were brought into the abattoir and once restrained were identified with a unique identifier (Figure 3.1). In Bamenda all cattle had a Tyvek® wristband, labelled with a unique identifier code, placed around the base of 1 ear. Head's of slaughtered cattle were kept with the carcass until meat inspection hence individual cattle were able to be identified throughout the slaughter process (Figure 3.1). In Ngaoundere abattoir all the butchers were given plastic meat inspection tags labelled with a unique identifier code to identify the head, pluck and liver of the carcass upon meat inspection (Figure 3.1). While the animal was restrained individual animal data was recorded including the animal's sex, breed and where the animal originated from the butcher. "Mixed breed" cattle were defined as cattle with mixed *Bos indicus* breeding. "Exotic" cattle defined as *Bos taurus* or a mix of *Bos taurus* and *Bos indicus* breeds. The dentition score (DS) was recorded for age of each bovine relating to the number of permanent incisor teeth present. Scores included 0 (No permanent incisors; <2 years), 1 (One permanent incisor;>2 and <2.5 years), 2 (Two permanent incisors; >2.5 and <3 years), 3 (Three permanent incisors; >3 and <4 years), 4 (Four permanent incisors; >4 years) and 5 (All four incisors in wear/ broken; 5+ years) (337). Body condition score (BCS) was was also recorded using a predefined scoring

system (0-5) with 0 being emaciated and 5 being obese (338). All individual animal data was recorded on a paper slaughter form for each animal as shown in appendix C. A veterinarian took up to 6ml of heparinised and coagulated blood samples from the jugular vein or tail vein in pre-labeled 6ml vacutainers. Cattle were then slaughtered, butchered and the carcass was presented for meat inspection by MINEPIA meat inspection staff. Any TB or fasciolosis lesions identified by a meat inspector were recorded on the animal's slaughter form.

Tuberculous lesion location and severity were recorded for up to 3 lesions per animal (Section 3.3.4). A sample of up to 3 TB lesions per animal was taken aseptically for further culture (Section 3.3.5). A small subset of non-lesioned cattle had a sample taken from a retropharyngeal LN for further culture. Further mycobacterial genotyping was conducted as part of a larger bTB epidemiological project in Cameroon. Subsequently the results beyond positive culture will not be included in this thesis. Any further reference to molecular work part of the larger bTB study will be acknowledged published papers, to the thesis of N. F. Egbe (University of Calibar, Nigeria) or referring to un-published work.

Fasciolosis lesions were also graded in severity with the protocol described (Section 3.4.2). Where possible samples of whole adult *Fasciola spp* parasite samples were taken from infected animals. Sampled animal information initially recorded in paper format was transferred to a purposely designed Microsoft Access® database within 2 days of collection. Additionally, due to the different organisational structure of Ngaoundere abattoir and time pressures additional veterinarians conducted sample collection as stated in the declaration of this thesis.

Biological samples

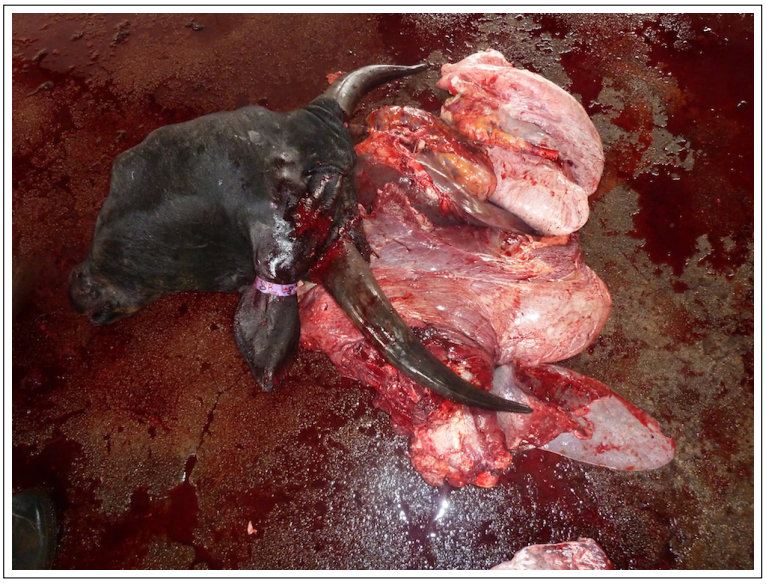
Various biological samples taken during the abattoir sampling for further diagnostic testing. The types of sample are as follows:

- Heparinised blood samples stored between 10° C to 26° C were cultured within 12 hours in the IFN-γ assay (Bovigam®) (Section 3.3.1).
- Plain blood samples were stored between 10° C to 26° C for up to 24 hours to allow blood to clot. The vacutainers were centrifuged at 3000g for 10minutes at room temperature (22° C +/-5° C) to separate the serum. After centrifugation 0.5-1.5ml of serum per vacutainer was transferred into a pre-labelled 1.8ml cryovial and stored at -20° C. Serum samples were heat treated at 56° C in a waterbath. Serum samples were then transported to the UK, continued to be stored at -20° C, and stored at the Roslin Institute, Royal (Dick) School of Veterinary Studies, Edinburgh, UK for testing. The bTB and fasciolosis ELISAs are described in sections 3.3.3 and 3.4.1 respectively.
- Whole adult *Fasciola spp* parasites were washed in sterile Phosphate buffered saline (PBS) 6 times, using a 2ml pasteur pipette, to visually remove blood and fragments of liver. Parasites were then stored in 95% ethanol at -20° C and labelled according to abattoir. A sample of whole adult *Fasciola spp* parasites were speciated by PCR as described in section 3.4.1.

(a) A typical cattle slaughtering environment in Bamenda abattoir (Bamenda, North West Region, Cameroon).

(b) Identification of cattle pre-slaughter (Bamenda, North West Region, Cameroon).

(c) Identification using ear bands on head displayed with offals for meat inspection (Bamenda, North West Region, Cameroon).



(d) Identification of livers using identification bands and butchers identification using lacerations (Ngaoundere, Vina Division, Cameroon).

Figure 3.1: **Images of abattoir fieldwork across Cameroon in 2012-13.**

3.2.2 Cross-sectional studies

Study sites

Two cross-sectional studies were conducted in pastoral and dairy cattle populations. Pastoral cattle were sampled from herds in two administrative areas of Cameroon; the NWR and the VD within the Adamawa Region (Figure 2.1). Dairy cattle were only sampled from dairy cooperatives in NWR where the majority of dairy farmers are. Due to the organisational differences in pastoral and dairy farmer communities described in Chapter 2; the populations were considered as two separate cattle rearing populations and sampled separately.

Study designs

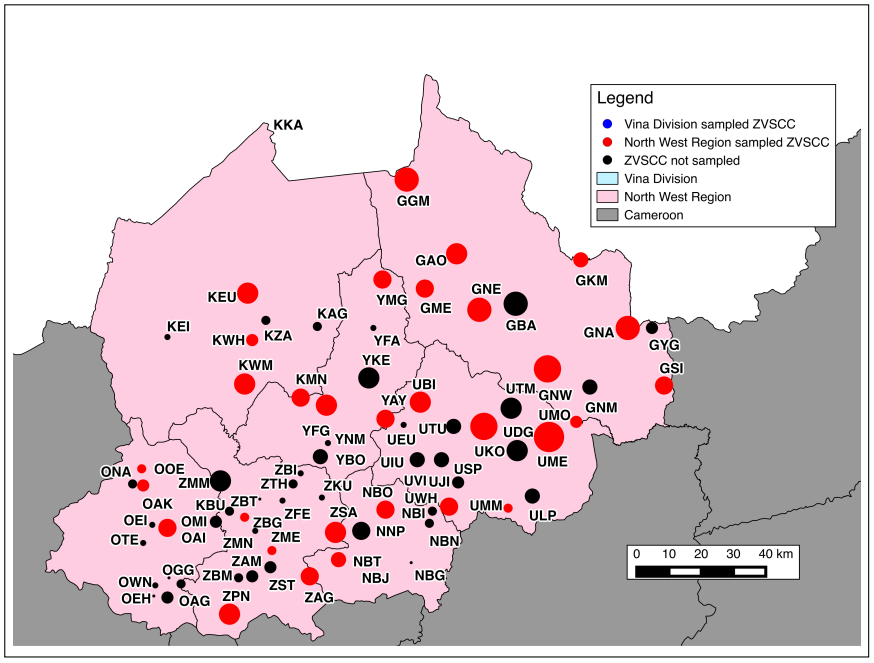
Cross-sectional studies were conducted when pastoral herds were unlikely to be undertaking transhumance; the NWR pastoralist and dairy farmer sampling was conducted January-May 2013 and VD pastoralist sampling was conducted September-November 2013. The target pastoralist populations were those herds listed in vaccination records in 2012 at 81 VCs in the NWR and 31 VCs in the VD. There were 5,053 herds in the NWR and 1,927 in the VD with a range of 1-215 cattle per herd (Appendix A). Sample sizes were calculated with the aim of using the SCITT to estimate the prevalence of bTB. They assumed a bTB prevalence of 50%, the SCITT to have a sensitivity of 60% and specificity of 90% and estimates of prevalence to be made with 95% confidence , 5% precision and took into account animal clustering (Survey Toolbox; AusVet) (336). This gave 1399 pastoralist cattle; rounded to 1500 for ease of selection. A stratified random sample of 1500 cattle was selected with the aim of sampling 15 animals from 100 different pastoralist herds. The list of herds in each site was stratified by administrative area; 7 Divisions in the NWR and 8

sub-Divisions within the VD and a random sample of 50 herds was taken from each site. The number of herds sampled per strata was proportional to the total number herds listed in these areas (Figure 3.2).

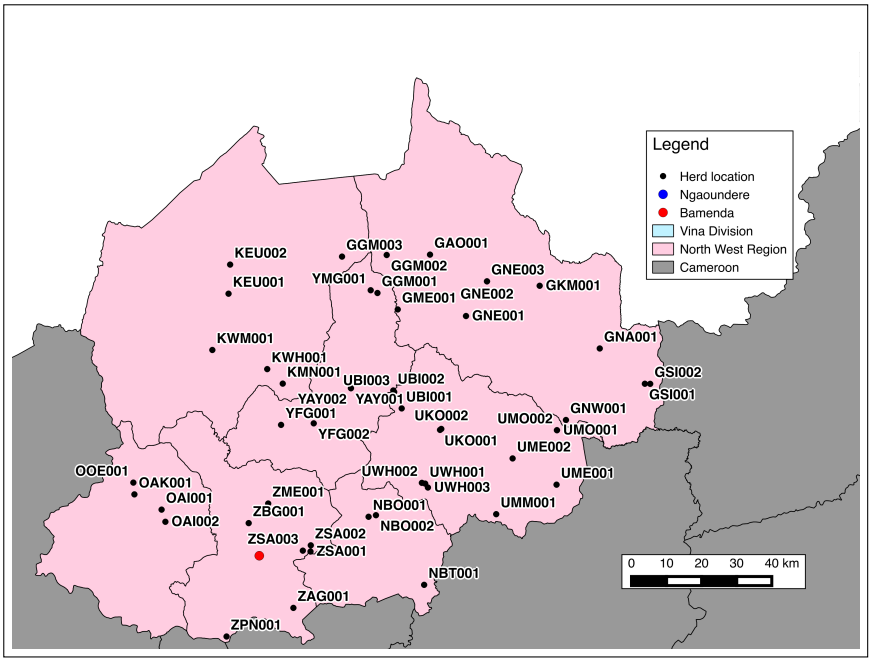
The small-scale dairy farmers were all registered with MINEPIA and address list for 2012 was obtained from their NWR office in Bamenda. These herds were established as part of an non-governmental organisation (NGO) initiative in the 1990s to improve milk production in the area by the importation of donated Holstein-Friesian cattle to be reared in zero grazing system (339). There were 229 dairy farmers, grouped into 7 cooperatives with seven to 95 farmers per cooperative. For logistical reasons, only the three largest cooperatives (164 farmers 492 cattle) were included. A simple random sample of 84 cattle was selected using the same criteria described for pastoralists. It was assumed that each dairy farmer would have 2 cows, so 46 farmers were selected proportional to the number in each cooperative (Figure 3.2).

Selected pastoralists and dairy farmers were contacted by phone or in person by the Head of the local VC, and asked if they wished to be involved in the study. Individuals were replaced by resampling if they declined to take part in the study, had died, moved out of the region, or were located more than three hours walk from a point that could be accessed by off-road vehicle, motorbike or on horseback.

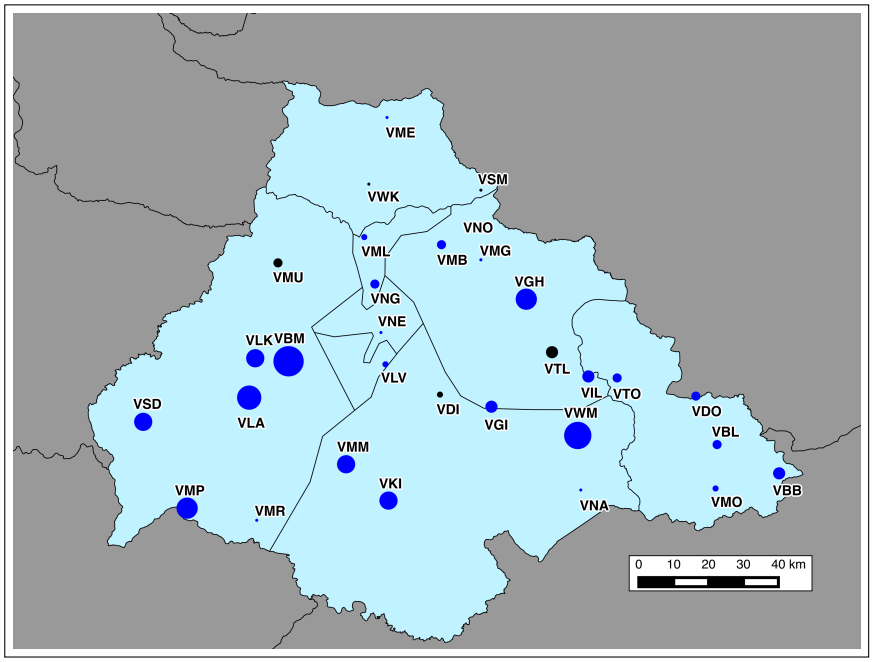
(a) North West Region VC locations with each point size proportional to the number of herds registered at that VC. VC coloured red had herds sampled in the study.



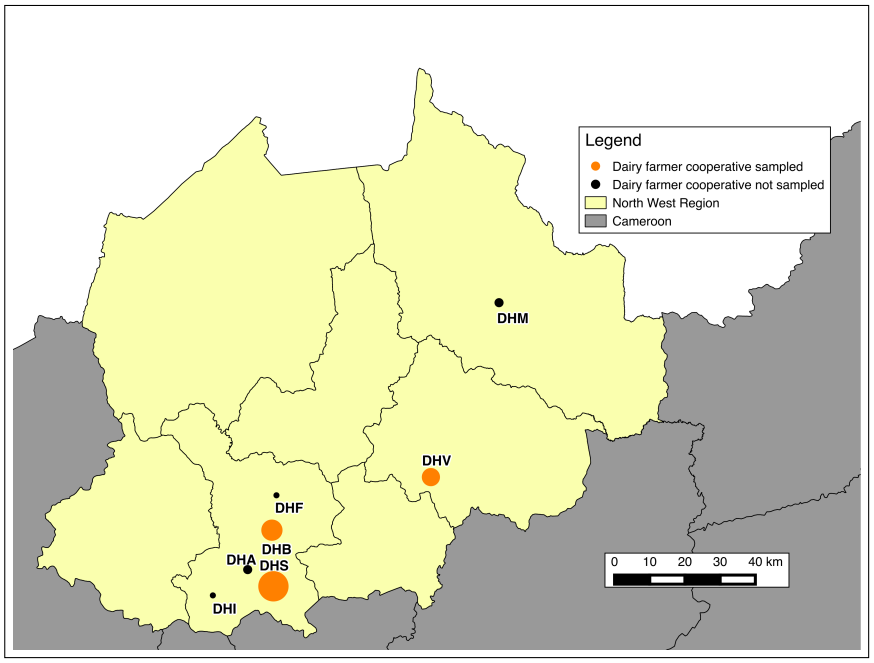
(b) North West Region sampled herd locations with herd ID.



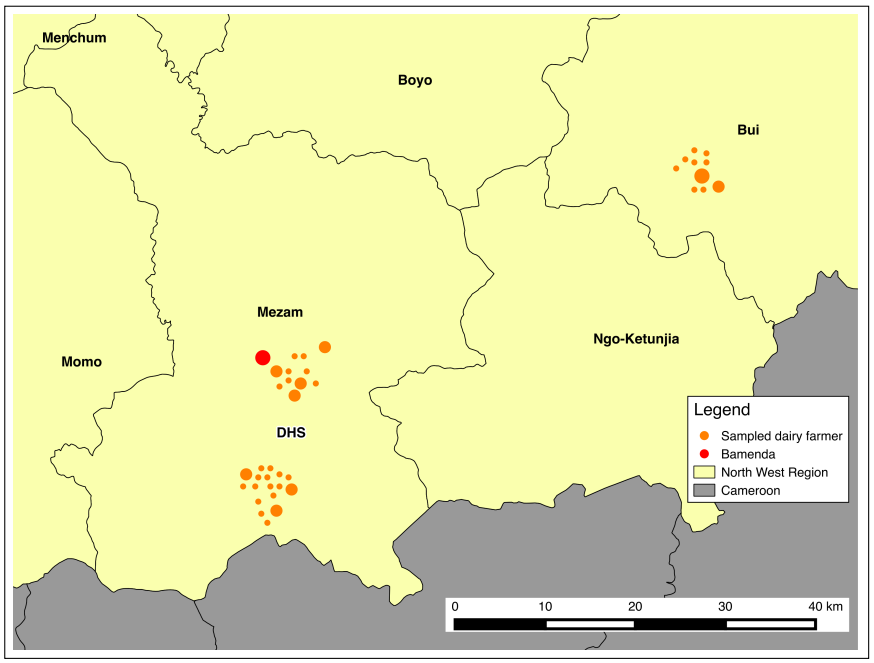
(c) Vina Division VC locations with each point size proportional to the number of herds registered at that VC. VC coloured blue had herds sampled in the study.



(d) Vina Division sampled herds locations with herd ID.



(e) Dairy farmers (in the North West Region) cooperative clusters locations with each point size proportional to the number of herds registered at that cluster. Clusters in orange were sampled in the study.



(f) Dairy farmers (in the North West Region) sampled herd locations within the 3 clusters with the size of each point proportional to number of cattle sampled (1-4 cattle).

Figure 3.2: **Location of sampled VC, pastoralist and dairy farmers herds in the North West Region and Vina Division, Adamawa Region, during 2013.**

Sampling methodology

Pastoralist herdsmen were visited either at their homestead or a convenient location in the vicinity and dairy herds were visited at the homestead. The same interviewer/ translator (Hamman Saidou Mustaffa) explained the project in either Fulfulde, Pidgin, English or French and verbally confirmed that the herdsman or farmer understood and was prepared to participate. He then administered, a structured herd level questionnaire by interview in the respondents preferred language. The questionnaire was developed through discussions with pastoralists, veterinary professionals and researchers. The questionnaire was pretested with pastoralists and veterinary professionals then modified prior use in the study. The questionnaire took 20-30 minutes to administer and was designed to collect data on cattle husbandry, dairy practices and knowledge of three infectious diseases: bTB, fasciolosis (Liver fluke), and Foot and Mouth disease (FMD) using local names where appropriate. These same names were used in Cameroonian Pidgin. The names "Soharu", "Balki" and "Njobu" were used in Foulfulde and "Tuberculose du bovine", "Douve du foie" and "FiÃĺvre aphteuse" in French. Awareness of an infectious disease was defined as "the participant recognising the name of the disease". If pastoralists or dairy farmers were not aware of a particular infectious disease no more questions were asked relating to the disease. Number of cattle presented and GPS location (Garmin eTrex® Venture) were also recorded.

From each herd 15 cattle were to be randomly sampled by the same local translator. The local translator was unaware of individual animal's health status and selected cattle nearest to him. Cattle randomly sampled, from each herd, were equally sampled into 3 age groups dependant upon the cattle presented. These age groups were calves (<1 years), youngstock (1-3 years) and adult cattle (3+ years). Dependant upon

handling facilities at the location each animal was caught and usually cast onto the ground. The animal was examined by the same veterinarian (R. F. Kelly) who also conducted all further tests and sampling. The animal was then identified with 2-3 Tyvek®wristbands; 2 placed around the uppermost forelimb above the hoof and if present 1 around the uppermost horn (Figure 3.3). Each wristbands was coded with a unique identifier code. While the animal was restrained the local translator would take a structured history of the animal from the cattle keeper and the animal. All individual animal data was recorded on a paper animal form for each herd by the local translator (Appendix F). Including information signalment, if it was presenting with specified clinical signs and if anthelmintic treatment had been administered in the previous 12 months. Occurrence of mastitis or abortion in the previous 12 months were recorded for female cattle older than 1 year of age. The predetermined list of clinical signs was devised from mind-maps, with researchers and veterinarians, focusing on clinical signs of bTB and fasciolosis. Signalment data included sex, breed, age and body condition score. "Mixed breed" cattle were defined as cattle with mixed *Bos indicus* breeding. "Exotic" cattle defined as *Bos taurus* or a mix of *Bos taurus* and *Bos indicus* breeds. The DS of sampled cattle was recorded for age of each bovine relating to the number of permanent incisor teeth present using the same method as the abattoir study (337). The BCS of sampled cattle was also recorded using a predefined scoring system (0-5) using the same method as the abattoir study (338). Plain and heparinised blood samples were collected in addition to the SCITT being conducted and skin thickness at both skin sites was recorded on the individual animal form (3.3.2 and figure 3.3).

A return visit, 3 days later, was scheduled to interpret the SCITT. Each animal was restrained an examined for any reaction to injected tuberculin at both sites. Any reactions were measured using manual bovine skin callipers, recorded on the

individual animal form, and Tyvek® wristbands were removed. Feedback was given at this point and veterinary assistance was offered free of charge to any cattle presented by the veterinarian. Questionnaire responses and clinical histories were initially recorded in paper format and transferred to a Microsoft Access® within seven days from collection (Figure 3.3).

Biological samples

Various biological samples taken during the cross-sectional sampling for further diagnostic testing. The types of sample are as follows:

- Heparinised blood samples stored between 10° C to 26° C and were cultured within 12 hours in the IFN-γ assay (Bovigam®) (Section 3.3.1).
- Plain blood samples were stored between 10° C to 26° C for up to 24 hours to allow blood to clot. The vacutainers were centrifuged at 3000g for 10minutes at room temperature (22° C +/-5° C) to separate the serum. After centrifugation 0.5-1.5ml of serum per vacutainer was transferred into a pre-labelled 1.8ml cryovial and stored at -20° C. Serum samples were heat treated at 56° C in a waterbath. Serum samples were then transported to the UK, continued to be stored at -20° C, and at the Roslin Institute, Royal (Dick) School of Veterinary Studies, Edinburgh, UK for testing. The fasciolosis ELISA is described in section 3.4.1.

(a) An example of transportation difficulties during sampling (Akeh, North West Region, Cameroon).

(b) Identification of cattle (Ndop, North West Region, Cameroon).

(c) The veterinarian (R. F. Kelly) taking blood samples from cattle during sampling (Sabga, North West Region, Cameroon)

(d) Local translator (S. M. Hamman) providing feedback on a return visit (Belel, Vina Division, Cameroon).

Figure 3.3: **Images of cross-sectional fieldwork across Cameroon in 2013.**

3.2.3 Ethics statement

The study design and sampling methodology was reviewed and approved by the University of Edinburgh Ethics Committee, UK (ERC No: OS02-13) and by the Institute of Research and Development (IRAD), Cameroon. IRAD gave permission to conduct the fieldwork and issued fieldwork permits. The research did not involve endangered or protected species and no further approvals were necessary to conduct fieldwork.

Verbal consent was granted to participate in the study and where appropriate for sampling to be conducted on private land. All participants had the purpose of the cross-sectional study explained to them, given opportunity to ask questions. All participants gave verbal informed consent to be involved and were aware they could opt out at any stage. Verbal consent was deemed appropriate for the variety of dialects spoken, variable literacy amongst participants and due to the remote outdoor fieldwork environment (334; 329). Information to be provided to participants, for informed verbal consent, was communicated to the translator/ interviewer (Hamman Saidou Mustaffa) in a written document. Additional training was provided to the interviewer regarding the consent procedure and interview process. Furthermore the interviewer was experienced in conducting questionnaires in similar studies and spoke the various local dialects of study participants (334). Verbal consent was recorded on a cover sheet to the questionnaire by the interviewer and refusals were recorded in separate document along with reasons for refusal.

3.2.4 Participant feedback

During cross-sectional study in 2013 pastoralists and dairy farmers were given the results of the SCITT for all sampled cattle on day 3 interpreted at >4mm. Also on day

3, regardless of the SCITT results, the zoonotic implications of bTB and protective public health measures were discussed with pastoralists and dairy farmers. In March 2015 research feedback was given to slaughter house and MINEPIA staff, via informal and formal presentations, in Bamenda and Ngaoundere. Letters were written to all VC staff, who participated in the cross-sectional study, about initial research findings. Furthermore a zoonotic bTB educational material was produced and distributed around abattoirs and VCs in February-March 2015 (Appendix G).

3.3 Bovine tuberculosis diagnostic tests

A combination of bTB diagnostic tests were performed in the cross-sectional and abattoir studies. The diagnostic tests conducted in the abattoir study were:

1. Commercial IFN-γ assay (Bovigam®).
2. Commercial *M.bovis* Antibody ELISA (IDEXX® *M.bovis* ELISA).
3. TB lesion identification and grading at meat inspection.
4. TB Lesion mycobacterial culture for *Mycobacterium tuberculosis complex*

The diagnostic tests conducted in the cross-sectional studies were:

1. Commercial IFN-γ assay (Bovigam®).
2. Single comparative intradermal skin test.

3.3.1 Commercial IFN-gamma assay

The IFN-γ assay is a commercial kit (Bovigam®) and was conducted as per published protocol (340; 341) in the LEID, University of Buea, Buea, South West Region,

Cameroon. Briefly within 12 hours of collection three aliquots of 1.5ml heparinised blood, per animal, were incubated with either 15ul of avian PPD , bovine PPD (Prionics® Lelystad Tuberculin PPD) or PBS for 24hours at 37° C. Samples were centrifuged at 300g for 10minutes, plasma aliquotted and subsequently stored at -20° C in portable travel freezer. Electrical supplies were maintained by mains electricity, portable generators or from vehicle batteries where necessary in the field. Plasma samples were transported at -20° C to Laboratory of Emerging Infectious Diseases (LEID), University of Buea, Buea, Cameroon to conduct the IFN-γ ELISA. Reagents were reconstituted where appropriate and samples were allowed to reach room temperature (22° C +/-5° C). The avian PPD, bovine PPD, and PBS previously stimulated plasma samples were diluted 1:1 with dilution buffer. Diluted plasma samples were added to the pre-coated 96 well plate along with duplicates of kit positive, negative and PBS controls. The 96 well plate was the then incubated on a microplate shaker, at 600RPM for 60 minutes at 22° C +/-5° C, and then washed 6 times with wash buffer. Conjugate was added to the 96 well plate incubated for 60 minutes and washed as previous. Enzyme was added to the 96 well plate incubated for 30 minutes as previous in the dark. Stopping solution to the 96 well plate and read at 450nm using an automated microplate reader (Thermoscientific® Multiskan Go). The acceptable averaged negative bovine was <0.13 and positive bovine control is >0.70. The difference in OD of the sample stimulated with bovine PPD minus the mean OD of the avian PPD was calculated for interpretation. At standard interpretation, as per commercial kit instructions, animals with a bovine PPD plasma sample of $\geq$0.1 that of avian PPD and PBS indicates the presence of *M.bovis* infection. A $\geq$0.05 can also be used as a severe interpretation. The interpretation of positive diagnostic cut-off values is discussed in chapters 4 and 8 of this thesis.

3.3.2 Single comparative intradermal tuberculin test

IFN-γrior conducting the SCITT the bovine was appropriately restrained by being either cast on the ground or tied to trees using ropes (Day 0). All SCITTs were performed and interpreted by the same veterinarian (R Kelly). Left or right side of the cervical neck was used, depending upon accessibility, and ID bands were placed on the leg and or horn on the same side of the bovine. Two areas, approximately 12cm apart, in the mid-cervical neck had the hair clipped using scissors to mark injection sites. Skin callipers were used to measure the skin thickness in both sites. Two multidose automatic syringes (McLintock textsuperscript®) were used to inject 0.1ml of avian and bovine PPD (Prionics® Lelystad Tuberculin PPD) injected intradermally in the dorsal and ventral sites respectively. The injection site was palpated to confirm PPDs were injected intradermally. Multidose automatic syringe needles were swabbed with surgical spirit between cattle. On a return visit, approximately 72 hours later (Day 3), the skin thickness of the two injection sites were measured using skin callipers. Results were recorded in the field using paper forms and transferred to a purposely designed Microsoft Access® database within 2 days of collection. The difference in bovine and avian PPD skin measurements were calculated for each bovine to determine if the animal was positive or negative for bTB. Firstly difference between the skin measurement of PPD injection sites on day 0 and 3 were calculated. Then difference between bovine and avian difference were then calculated:

$$Avian\ skin\ reaction\ difference\ (A)\ =$$

$$skin\ thickness\ day\ 3(mm)\ -\ skin\ thickness\ day\ 0$$

$$Bovine\ skin\ reaction\ difference\ (B)\ =$$
$$skin\ thickness\ day\ 3(mm)\ -\ skin\ thickness\ day\ 0$$

$$PPD\ skin\ reaction\ difference\ (S)\ =\ B\ -\ A$$

The interpretation of positive diagnostic cut-off values is discussed in chapter 8 of this thesis.

3.3.3 Commercial *Mycobacterium bovis* antibody ELISA

Serum samples were tested at the R(D)SVS, University of Edinburgh, Edinburgh, UK using a commercial *M.bovis* Antibody ELISA kit (IDEXX® *M.bovis* Antibody ELISA) as per published protocol (180). Briefly reagents were reconstituted where appropriate and samples were allowed to reach room temperature (22° C +/-5° C). Serum samples were diluted to 2:100 with dilution buffer and added to the *M.bovis* antigen pre-coated 96 well plate along with duplicates of the kit positive and negative controls. 96 well plates were incubated for 60 minutes at 22° C +/-5° C, and then washed 5 times with wash buffer. Anti-Bovine Horseradish peroxidase conjugate was added to the 96 well plates then incubated for 30 minutes at 22v +/-5° C, and then washed 5 times with wash buffer. TMB substrate was added to the 96 well plates then incubated for 15 minutes at 22° C +/-5° C, and then washed 5 times with wash buffer. Finally stop solution was added and the plates read at 450nm using an automated

microplate reader (Thermoscientific® Multiskan Go). The mean positive and negative control values are calculated and pass if the mean positive control is greater than or equal 0.3 and the negative is less than or equal to 0.2. The sample to positive ratio (S/P ratio) is calculated to determine if the sample is positive or negative. The equation is as follows:

$$S/P = \begin{cases} Sample\ OD - Mean\ negative\ control\ OD \\ \hline Mean\ positive\ control\ OD - Mean\ negative\ control\ OD \end{cases}$$

If a sample had a result greater or equal to 0.3 the sample is positive for bTB antibodies. If less than 0.3 the sample is considered negative for bTB antibodies.

3.3.4 Tuberculosis lesion identification and grading at meat inspection

After an animal has been identified in the abattoir study, sampled (Section 3.2.1) and slaughtered the carcass is examined for bTB by MINEPIA trained meat inspectors prior resale of meat. Meat inspectors vary on different sampling days and between the two abattoirs sampled. The meat inspectors examine the carcass systematically and identify gross lesions suspicious of bTB. An animal is reported as TB lesion positive if one or more "muco-purulent, caseous or calcified granulomatous tubercle" like lesions are identified in one or multiple tissues by the duty meat inspector. An animal is considered bTB lesion negative if no TB lesions are identified in any tissue by the duty meat inspector.

Once an bovine was identified to have one or more lesions up to three TB lesions

were graded by a member of the research team. Each lesion, which had been sliced open during routine meat inspection, was given a score for pathology, type, size, scale outlined in Table 3.1 and tissue affected was also recorded. These grading systems were adapted from a similar grading system used in a bTB epidemiological study in Ethiopia (96).

PATHOLOGY SCORE	
Score	**Definition**
0	No visible lesion
1	No gross lesion but apparent upon slicing
2	Less than or equal to 5 gross lesions
3	Greater than 5 gross lesions
4	Gross coalescing lesion
TYPE SCORE	
Score	**Definition**
0	No visible lesion
1	Mucoid/ purulent: Sticky, containing mucous like matter or consisting of pus.
2	Caseous: Necrotic/ soft cheese like substance.
3	Calcified: hard and containing calcium deposits.
SIZE SCORE	
Score	**Definition**
0	No visible lesion
1	Less than 10mm
2	10-50mm
3	Greater than 50mm
SCALE SCORE	
Score	**Definition**
0	No visible lesion
1	Single
2	Multiple

Table 3.1: **Bovine tuberculosis lesion scores.**

3.3.5 Tuberculosis lesion mycobacterial culture for *Mycobacterium tuberculosis complex*

Once graded all lesions graded were sampled for subsequent culture. Lesion samples were stored on ice and transported back to the field lab or the Tuberculosis Reference Laboratory (TBRL) Bamenda. The samples from the Ngaoundere were stored in liquid nitrogen and shipped to the TBRL, Bamenda, Cameroon in dry shippers. Upon arrival at the TBRL the lesion samples were either thawed and processed immediately or stored at -80° C until processed. Biohazard measures were taken during collection, handling and transportation of the specimens to avoid cross contamination and prevent exposure. Mycobacterial culture was conducted in the TBRL, Bamenda, Cameroon by Mr E. F. Nkongho (University of Calibar, Nigeria).

Briefly lesion samples were prepared and cultured as described by the World Organisation of Animal Health (OIE) with minor modifications (45; 7). All TB lesions and subsequent preparations were handled in Category 2 standard biosafety cabinets. Briefly all connective and fat tissue were removed from TB lesions and between 3-5g of lesion were sliced into <0.5cm using a sterile scalpel blade. Sliced lesion, mixed with 1-2g of sterile sand and 5ml of sterile 85% saline, was then ground into a paste using a sterile pestle and mortar. The paste was then transferred to a 50ml falcon tube and decontaminated by adding an equivalent volume of 4% NaOH and left to stand for 15 min and intermittently vortexed. Sterile PBS (pH 6.8, 0.067M) was then added up to the 50 ml mark and the tube was centrifuged at 3200g for 20 minutes at 18° C. The resulting pellet was re-suspended in 2ml of sterile PBS and 0.1ml (2 drops) was inoculated onto each of two slopes of Lowenstein Jensen (LJ) media (one supplemented with pyruvate and the other with glycerol). The LJ slopes were then incubated at 37° C. A third culture was carried out using a Mycobacterial Growth Indicator Tube (MGIT) containing a modified Middlebrook 7H9 broth with polymixin

B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin (PANTA)/OADC (Becton Dickinson Diagnostics, New York, USA) incubated at 37° C in an incubator (BACTEC960 automated incubator, Becton Dickinson Diagnostics, New York, USA). Inoculated LJ media were kept at at 37° C and examined once a week for up to 12 weeks for microorganism growth; if negative after 12 weeks the slopes were discarded. Inoculated MGITs were examined daily by the MGIT instrument electronically for positive growth for up to 8 weeks. If no growth was reported at 8 weeks the MGIT culture was examined manually for growth; negatives were then discarded. Any observed growth on the LJ medium or MGIT machine positive growth were scraped and used to prepare a suspension in 3% formal saline, heat-fixed on a hot plate at 75° C for 30 minutes, stained by the Ziehl-Neelsen (ZN) method (342) and viewed under the 100X objective of a light microscope to determine the presence and morphology of acid-fact bacteria (AFB). Negative samples suspected for AFB growth were also examined in a similar manner. Individual cattle were deemed TB culture positive if mycobacteria were cultured from one or more lesion samples in one or more culture techniques. Cattle with no mycobacterial growth for TB culture, using any of the culture techniques, were deemed TB culture negative. Cattle which had no TB lesions identified at slaughter were deemed unknown status for TB culture. Further genotyping was conducted by Mr E. F. Nkongho (University of Calibar, Nigeria) and the are techniques described in his PhD thesis.

3.4 Fasciolosis diagnostic tests

A combination of fasciolosis diagnostic tests were performed in the cross-sectional and abattoir studies. The diagnostic test conducted in the abattoir study was:

1. Fasciolosis identification at meat inspection.

The diagnostic test conducted in the cross-sectional studies was:

1. *Fasciola gigantica* antibody ELISA. *Fasciola gigantica* antibody ELISA development is discussed in (Chapter 6).

3.4.1 Development of in-house *Fasciola gigantica* antibody ELISA

A series of laboratory techniques were used to develop the *Fasciola gigantica* antibody ELISA and are subsequently described:

1. *Fasciola* species RAPD PCR
2. *Fasciola gigantica* excretory/ secretory protein (ESP) antigen collection
3. *Fasciola gigantica* antibody ELISA

Fasciola species RAPD-derived sequence PCR

In the field locations in Cameroon parasites were washed 6 times in sterile PBS to remove liver tissue, bile and blood. Parasites were then preserved in 70-100% ethanol at time of collection, stored at -20° C and subsequently transported to the UK for PCR speciation.

PCR speciation was conducted in the Veterinary Parasitology Department, University of Liverpool, Liverpool UK. *Fasciola* species-specific primer sets were used in two comparative speciation PCRs on each parasite sample. Subsequently for each PCR approximately 25mg of tissue was removed from the caudal portion of each parasite. Each sample was washed six times in sterile PBS and ethanol was allowed to

evaporate for two hours before genomic DNA was extracted using a DNeasy® blood and tissue kit (QIAGEN®, Crawley, UK) and used the same day.

PCR primers were produced by Dr J. McGarry, University of Liverpool from known *F. hepatica* and *F. gigantica* parasite specimens using RAPD analyses (223). Primer set one was derived from a *F. hepatica* parasite from the UK (Forward primer: 5'GCG GCC AAA TAT GAG TCA-3' and reverse primer: 5'-CTG GAG ATT CCG GTT ACC AA-3' of 568bp). Primer set two was from a Ghanaian *F. gigantica* parasite (Forward primer: 5'-GTT CAG GTG ACA AGC CAA-3' and reverse primer: 5'-ATC ACA CCG TGA AGC AGA-3' of 396-bp). Each PCR contained 12.5µl of BioMix®Red (1X, Bioline®, Sydney, Australia), 0.5µl of 10µmol forward primer from set one or two, 0.5µl of 10µmol reverse primer set one or two (final concentration of both primers 0.2µmol) , 10.5µl of sterile water and 1.0µl of template DNA (>10ng) from sampled parasites (Total volume 25µl). For the *F. hepatica* primer set the thermocycler (Biometra® T3 Thermocycler) PCR conditions were 95° C initial denaturation for 15mins; 30 cycles of 94° C for 30secs, 54° C for 40secs and 72° C for 1min; and a final 72° C 3min extension. For the *F. gigantica* primer set PCR the thermocycler was set at 95° C for 15mins; 30 cycles of 94° C for 30secs, 56° C for 40secs and 72° C for 1min; a final 72° C 3min extension. The positive *F. hepatica* control was from a cow in the UK (B2 lin6). Positive *F. gigantica* control was from Uganda (Ug2). A sample of bovine DNA and a sample of sterile water were used as negative controls in each series PCR and a 100 bp DNA ladder (GENEFLOW®) was used. PCR products were stained with 10µl in 100ml SYBRsafe® (1X, Life technologies®) and separated in 1.5% agarose gel for 45mins at 150 volts. Separated PCR products were visualised in ultraviolet light (UV) by trans-illumination.

Fasciola gigantica ESP antigen collection

Antigen collection was undertaken in the TBRL, Cameroon. Live parasites were washed 6 times in sterile PBS and then 6 times with RPMI tissue culture medium to remove liver tissue, bile and blood. Parasites were then incubated in 1ml of RPMI medium for 12 hours at 37° C in a humidified atmosphere in a poultry hatching incubator (Hova-Bator® Incubator 37C60E). Subsequently the supernatant was collected and centrifuged at 10,000g for 30 minutes at 4° C to remove particulate material. Supernatants were further filtered by passing through individual 0.22Îijm filters (Sartorius, Minisart® 16532K). Samples were stored at -20° C in aliquots and transported to the UK refrigerated.

Fasciola gigantica antibody ELISA

Initial pilot development of the *F. gigantica* ELISA was conducted in Department of Veterinary Parasitology, University of Liverpool, Liverpool UK. Final development and serum sample testing was conducted in R(D)SVS, Edinburgh, UK. Firstly Immulon-2 ELISA 96-well plates were coated with 100ul of 1ug/ml *F. gigantica* E/S antigen in 0.1M carbonate buffer (pH 9.6). Plates were incubated for 1 hour at room temperature and then refrigerated at 2-4° C overnight. Plates were then washed 6 times (2 short washes and 1 5 minute wash repeated twice) with pH 7.2 PBS containing 0.05% Tween-20 (PBS-Tween). Each well was then blocked with 200ul of blocking buffer for 1 hour at 37° C (4% skimmed powder (Marvel, Premier International Foods®, Spalding UK) in PBS-Tween). Plates were then washed six times, in the same manner as previously stated, and 100ul of diluted 1:200 test sera and blocking buffer. The positive control used was sera collected from an animal identified in Bamenda abattoir positive for *F. gigantica* infection at meat inspection and PCR. A *Fasciola* species negative cow, which had been kept indoors throughout her life at Ness Heath Research Farm (University of Liverpool, Cheshire UK), was used at as the negative control sera. Positive and negative controls were added to the plate in duplicate, at the same concentration as the test sera, and incubated at 37° C for 1 hour. The plates were again washed and 100ul of 1:1500 mouse anti-bovine IgG HRP conjugate (Serotec®, UK) and blocking buffer again incubated at 37° C for 1 hour. After washing 100ul of TMB substrate (Acetate buffer pH 5 and tetramethylbenzidine in a methanol based solution, MAST Diagnostics, Bootle, Merseyside, UK) was added and incubated at room temperature for 20 mins in the dark. Finally 100ul of stopping solution (10% Hydrochloric acid) was added and the colour change measured at 450nm using an automated microplate reader (Thermoscientific® Multiskan Go). The results were obtained as an OD and

expressed as a percent positive (PP) value:

$$PP = \frac{OD\ of\ test\ sample}{Mean\ OD\ of\ positive\ control} \times 100$$

If a sample had a result $\geq$12.8% the sample is positive for *F. gigantica* antibodies. If less than 12.8% the sample is considered negative for *F. gigantica* antibodies. Two plates at the start and end of each week were duplicated to assess for operator consistency. If plates differed in result they were repeated. Selection of the $\geq$12.8% positive cut-off value is described in chapter 6.

3.4.2 Fasciolosis lesion identification at meat inspection

In the abattoir study after an animal has been identified, sampled (3.2.1) and slaughtered the carcass is examined for fasciolosis by MINEPIA trained meat inspectors prior resale of meat. Meat inspectors vary on different sampling days and between the two abattoirs sampled. The meat inspectors examine the liver systematically to identify fasciolosis gross pathology by slicing down the common bile duct with an additional 1-2 slices through the liver parenchyma. An animal is reported as fasciolosis lesion positive if gross pathology for fasciolosis was identified by the duty meat inspector. An animal is considered fasciolosis lesion negative if no fasciolosis gross pathology was identified in the liver by the duty meat inspector.

Once an animal was identified to have gross *Fasciola* pathology in the liver it was graded by a member of the research team. Each positive liver, which had been sliced open during routine meat inspection, was given a pathology score outlined in Table 3.2. This pathology score was adapted from a similar score used in a fasciolosis study in a Belgian abattoir (239). Throughout this thesis the *Fasciola* pathology score is recategorised to "Negative (0)" and "Positive (1-3)" due to discrepancies in scoring between sampling teams.

Pathology score	
Score	**Definition**
0	No visible pathology
1	Low grade pathology: minimal damage to the parenchyma of the liver through migratory fibrotic/ cirrhotic tracts from the parasite, thickening of bile ducts with a few *Fasciola* species parasites noted in bile ducts.
2	Moderate grade pathology: *Fasciola* species parasites found in the bile ducts and up to approximately half the liver has evidence of fibrosis/ cirrhosis.
3	Severe grade pathology: The majority of the liver is noted to have extensive fibrosis/ cirrhosis without having to cut the surface of the liver.

Table 3.2: ***Fasciola* species infection pathology score.**

3.5 Statistical analyses

This section describes the statistical methods used frequently throughout the thesis. Methods used in an individual chapter are described further in the chapter. All statistical analyses in this thesis were performed using packages and functions in R Studio 0.98®(343). All graphs were produced using the *ggplot2* package (344). Maps were drawn using QGIS 2.2® (345) and shape files obtained from the open access GADM database of Global Administrative Areas (www.gadm.org).

3.5.1 Basic statistical analysis

Descriptive statistics were used to describe samples from the abattoir and cross-sectional studies. Point estimates such as means, medians, proportions and confidence intervals were used to describe samples. Confidence intervals (CI) are at 95% level throughout unless otherwise stated. The stratified design of the pastoral cross-sectional study was incorporated into descriptive analysis, to account for stratification of sampling by Division (NWR) or Sub-division (VD) by herd and clustering of animals by herd, using the the *svydesign* function in the *survey* package (346) where applicable. Sample statistics were estimated using *svymean*, *confint* and *svyby* functions. In each chapter specific differences in sample statistics are identified by non-overlapping CIs unless specific hypotheses are tested (347).

Where specific hypotheses are tested, the students T test was used to compare mean values where samples are considered independent of one another (348). The formula is outlined below:

$$t = \frac{\bar{x}-\mu_0}{s/\sqrt{n}}$$

Where proportions are compared, from independent samples, are considered the Chi

squared test is used (348):

$$\chi^2 = \Sigma \frac{(O_i - E_i)^2}{E_i}$$

Respectively the *t.test* and *chisq.test* function are used in the *stats* package (343).Unless otherwise stated results were significant where the p value$\leq$0.05.

Principle components analysis

Principal components analysis (PCA) reduces continuous measures to fewer principal components which are linear combinations of the original variables (349). Hence the technique reduces high dimensional data to orthogonal components to try explain the variation in the original variables. The first principal component describes the axis of most variation, with each subsequent component explaining the maximum of the remaining axes of variation. It can allow the covariance of several continuous measures and the inter-relationships between different parameters to be diagrammatically represented in a PCA plot. It does not necessarily separate sub-groups within samples (349) but may do if they are substantially different in variation. However, the output from PCA describes how much each of the original variables contributes to each of the summary components. The *princomp* function in the *stats* package (343) was used to calculate PCA and PCA plots were created using *ggbiplot* function in the *ggplot2* package to demonstrate the variance explained by principle components (350). The function allows the incorporation of both quantitative and qualitative supplementary variables. In chapter 4 PCA is used to calculate an overall lesion score (OLS) for TB lesions, using 4 different scoring methods which measure different aspects of lesion pathology, in an attempt to quantify lesion severity. The principle component which explains the most variation between the 4 different scores is used to calculate the OLS.

0.5cm

3.5.2 Diagnostic test performance

Sensitivity, specificity and predictive values

Diagnostic test performance can be estimated relative to a gold-standard result and the results quantified as sensitivity or specificity of the diagnostic test. Sensitivity is defined as the proportion or percentage of true positives (Defined by the gold-standard diagnostic test) identified as test positive. Specificity is defined as the proportion or percentage of true negatives (Defined by the gold-standard diagnostic test) identified as test negative. Hence sensitivity also provides an impression of the proportion of expected false negative results and specificity of false positive results (134). Sensitivity and specificity, including 95% CI, were calculated in this thesis using the *epi.tests* function in the *epiR* package (351), unless otherwise stated, using the following equations:

	Test +	Test -
Disease +	a (True positive)	b (False negative)
Disease -	c (False positive)	d (True negative)

Table 3.3: **A generic 2x2 table comparing results from two diagnostic tests.** *Total (n)*= $a + b + c + d$ (134).

$$\textit{Sensitivity (SE)} = a \,/\, a + b$$
$$\textit{Specificity (SP)} = d \,/\, c + d$$

Due to no gold standard diagnostic tests being used in these studies, hence no test was being 100% sensitive or specific, for bTB or *Fasciola* diagnosis, calculation of sensitivity and specificity should be considered in light of the test being used to define "true" disease status. Sensitivity and specificity are inversely related and by changing the positive cut-off value of the diagnostic test will affect their magnitude (352). Hence selection of the positive cut-off value will depend upon the purpose of the diagnostic test; whether to maximise SE, SP or maintain both. Selection of a positive

cut-off value, based on SE and SP, can be conducted using receiver operating characteristic analysis (353; 354). At defined positive cut-off values the sensitivity, specificity and associated 95% CI of a diagnostic test is calculated using the *epi.test* function in the *epiR* package (351). Where a gold-standard diagnostic test does not exist SE and SP can still be estimated using non-gold standard Bayesian methods (134). The results from non-gold standard Bayesian analysis, conducted by M. Bronsvoort and I. Handel, is discussed in chapters 4 and 8.

The characteristics of the diagnostic test can be quantified by calculating sensitivity and specificity. Predictive values quantify for an individual animal within a population the probability of a test correctly identifying its disease state (Positive or negative). Specifically the positive predictive value (PPV) is the proportion of test positive animals that truly have disease. Conversely the negative predictive value (NPV) is the proportion of test negative animals that truly do not have disease (355). The following equations define PPV and NPV:

$$\textit{Positive predictive value (PPV)} = (TP \times SE) / ((TP \times SE) + (1-TP) \times (1-SP))$$
$$\textit{Negative predictive value (NPV)} = ((1-TP) \times SP / ((1-TP \times (SP)) + TP \times (1-SE)))$$

Key: TP= True prevalence; SE= Sensitivity; SP= Specificity.

Calculation of PPV and NPV is undertaken using the *epi.test* function of the *epiR* package (351). It is worth noting that predictive values are affected by prevalence of disease as well as sensitivity and specificity of the diagnostic test. Hence predictive values are not suitable to quantify and compare diagnostic test performance in populations with differing prevalences. However altering the positive cut-off value of the diagnostic test, to increase test specificity, will increase a test's PPV. This might be advantageous in when trying to identify disease positive animals (134).

Receiver operating characteristic analysis

As sensitivity and specificity have an inverse relationship adjusting the positive cut-off point of the diagnostic test will favour one over the other. For example increased specificity (Fewer false positives) or sensitivity (Fewer false negatives) maybe favourable in a diagnostic testing strategy. Receiver operating characteristic (ROC) analysis is useful for selecting a diagnostic cut-off depending upon the desired qualities of the diagnostic test (353; 354). The technique is based upon a predictive model that classes known positive and negative samples as test positive or negative at all possible positive cut-off values for the diagnostic test. The calculated sensitivity and 1-specificity (False positive rate), at the various positive cut-off points, is plotted as a ROC curve. Points for positive cut-off values lying above a diagonal line, drawn with an intercept of zero and a gradient of one, denote the diagnostic test is performing better than chance guess at a diagnosis. The point closest to the top left corner of the plot demonstrates the optimal positive cut-off value to maintain optimal sensitivity and specificity. This can also be calculated by the area under the curve (AUC) representing the probability that the diagnostic test will be better at diagnosing disease than chance alone (>0.5). In this thesis a probability of $\geq$0.8 implies that a diagnostic test had the ability to discriminate between diseased and non-diseased animals (134). In chapter 6 the ROC analysis is calculated and ROC curve drawn using the *roc* and *plot* functions in the *pROC* package(356).

Agreement analysis

Agreement analyses are used in this thesis to compare the performance between diagnostic as no gold-standard diagnostic tests were available. Agreement is defined as how much two diagnostic tests, which measure the same response or substance,

agree with each other (134). Agreement between diagnostic tests is quantified using various statistical methods for continuous and categorical data types. Furthermore in the absence of gold-standard diagnostics, agreement analysis is used in this thesis to determine the positive cut-off values for diagnostic tests with continuous results to be converted to categorical results (e.g. Positive and negative). Agreement between diagnostic tests was repeated at various positive cut-off values to determine the cut-off value to be used to describe bTB epidemiology.

For continuous scale diagnostic test results Spearman's correlation coefficient is used to investigate agreement, through measuring association, between diagnostic tests with numeric outputs. Such as raw diagnostic test results from the SCITT or IFN-γ assay. Spearman's correlation coefficient was calculated as diagnostic test are likely to have a monotonic, rather than linear, association and cannot assumed to be normally distributed (348):

$$\textit{Spearman's correlation coefficient } (r) = 1-(6\sum d^2 / n^3 - n)$$

Key: d= difference between ranks ; n=sample size.

The Spearman's correlation coefficient is interpreted on a scale of +1.0 (Perfect positive association) to -1.0 (Perfect negative association). Spearman's correlation coefficient is used to determine whether the was a correlation between two numeric results using *cor* function in the *stats* package (343). If a correlation was present the statistical significance of the correlation was tested at a significance level of $p<0.05$ using *cor.test* function in the *stats* package (343).

For categorical diagnostic results (e.g. Positive or negative) percentage agreement was used as a provisional measure of agreement between two diagnostic tests (134). The calculation is outlined below:

$$\textit{Percentage agreement or observed agreement } (OP) = (a + d/\, a + b + c + d) \times 10$$

However percentage agreement does not distinguish agreement between positive or negative diagnostic test results and does not adjust for chance. Cohen's kappa statistic was also calculated to determine the level of agreement, beyond chance, between two categorical diagnostic tests (e.g. Positive or negative) (357). To calculate Cohen's kappa statistic for two diagnostic tests the test should be independent of each other:

$$\textit{Expected agreement (EP)} = (((a + b)(a + c))/n + ((c + d)(b + d))/n)/n$$
$$\textit{Cohen's kappa statistic } (\kappa) = (OP\text{-}EP)/(1\text{-}EP)$$

Overall the effect of very low or high prevalence is thought to have a negligible effect on the calculation (358). Cohen's kappa statistic is interpreted in this thesis as =1 (Perfect agreement), 0.81-1 (Almost perfect agreement), 0.61-0.8 (Substantial agreement), 0.41-0.6 (Moderate agreement), 0.21-0.4 (Fair agreement), 0.01-0.2 (Poor agreement), $\leq$0 (No agreement) (352). Percentage agreement and Cohen's kappa statistic were used to quantify agreement between two tests. The *agree*, *kappa2* and *rater.bias* in the *irr* package (359) were used to calculate percentage agreement and Cohen's kappa statistic.

3.5.3 Regression models

Logistic regression is a type of generalised linear modelling, that predicts a binary outcome. For example the outcome could be whether to predict whether an animal is "diseased" or not "diseased" by a exposure variable or risk factor. Univariable logistic regression modelling only takes into account one exposure variable that may be associated with a given outcome. Multivariable logistic regression (MLR) models estimate the probability of the outcome given more than one categorical and continuous exposure variables and may take into account interaction between exposure variables. In non-experimental situations usually there are multiple variables

which are associated with a given outcome (348). Hence in this thesis MLR models were used to identify variables associated with a given outcome rather than univariate logistic regression models A generic logistic regression model is defined as:

$$logit(Y) = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + ...\beta_i X_i + \mu + \varepsilon$$

Model key: Y= the probability of the binary outcome ; β*= the fixed effects; X= the covariates;* μ*= the random effects;* ε*= the error.*

Categorical and continuous variables are used to generate a linear predictor, which is then transformed to the probability scale using the logit transformation. This transforms a predictor probability from a scale of -∞ to +∞ to a value between of 0 to 1. The exponential of β produces an odds ratio (OR), including OR CI, for the change in the predictor variable (X) and its significance determined by a p value (360). All MLR models were mixed-effect models including both fixed and random effects. Random effects are an adjunct method within model building to account for the hierarchical structure of the sampled data, minimise the over-estimation of sample size and correct for uncertainty. For example repeated sampling of cattle in the same area or herd are more likely to be more similar than in other herds or areas. This was to attain, in both the abattoir and cross-sectional studies, a representative sample throughout the geographical locations and the geographical locations were not specifically of interest but representative of the study area (NW and VD). Hence random effects were included in MLR models to take into account sample clustering within the study designs (Section 3.2).

For continuous variables to be included in a MLR model they have to have a linear relationship to logit(Y). If this is not true continuous variables need to be categorised for inclusion in the model (361) to capture the non-linear relationship within the

model (361). Linearity can be tested using the Box-Tidwell test where logit(Y) is modelled by an interaction between the continuous variable and the log of that variable. If the interaction term is statistically significant this suggests that linearity is not present (360).

In this thesis two types of selection are used for final logistic model selection are outlined with specific differences outlined in each chapter. In chapters 4, 5 and 8, MLR model selection is based on a conservative approach where all possible explanatory variables in final models and selection compares different interactions between variables (360). Model selection was based on the Akaike information criterion (AIC) and the final model was selected using the lowest AIC. The AIC is a comparative assessment of a model, compared to the other models, for a the sample data tested. By means of comparison the AIC will help select the most parsimonious model to fit the data and penalise for model fit in order to select the final model. The AIC is calculated using the following formula:

$$\textit{Akaike information criterion (AIC)} = -2InL + a \times s$$

Key: InL= is the log-likelihood function of the model; a= penalty constant; s= 1 + the number of explanatory variables in the model (360)

Final model selection was verified by computing AIC and ΔAIC. ΔAIC is more appropriate for small sample sizes in models where n$\leq$100 (362). AIC and ΔAIC were calculated using the *AICcmodavg* package and *modavg* function (363).

In chapters 7 and 8, backwards stepwise model selection is used where there are a larger number of possible explanatory variables that could be included in model selection:

1. Univariable logistic regression was used to screen explanatory variables where there was $\geq$10 (361). Variables were screened that were deemed plausible for

each model. Correlation between variables was undertaken by calculating the phi coefficient using the *psych* package (364). If phi was $\geq$0.5 two variables were considered correlated and the explanatory variable with the highest p value was selected. Variables were included in final MLR model selection if their p value $\leq$0.2. If there were <10 explanatory variables of interest all were included in the final MLR model selection protocols.

2. A backwards stepwise approach was used to find the best fitting model to describe the dataset when constructing the model with assessment for interactions (360). Model selection was based on the Akaike information criterion (AIC) as described previously (360). Each variable from the model was removed singularly to assess changes in AIC until the lowest AIC was achieved. The p value, odds ratio with 95% CI for explanatory variables were also estimated. Explanatory variables were deemed significant in their OR CI did not include 1 and the p value$\leq$0.05.

Chapter 4

Performance of the interferon-gamma assay in comparison to bovine tuberculosis pathology and serology.

4.1 Introduction

Bovine tuberculosis is a chronic disease of cattle, caused by *M. bovis*, and a zoonosis associated with close interaction with cattle or consumption of raw dairy products (15). Specifically, close contact between cattle and people is commonplace in rural communities in Cameroon (Chapter 2) and it is vital to understand the potential impact of bTB and the public health risks in such communities. Assessing bTB epidemiology can help to quantify disease in cattle, by estimating bTB prevalence, but estimates rely on the performance of the diagnostic tests used. Currently, identifying TB lesions at abattoir meat inspection is the only routine diagnostic test conducted in Cameroon. Identifying characteristic TB lesion pathology has been used to describe the epidemiology of bTB in abattoirs in many African countries (365; 48) including Cameroon (114; 75; 115; 116). Specificity of lesion detection is high (>95%) and can be improved further with culture of lesions or by using molecular techniques such as PCR to characterise *M. bovis* bacilli (138; 45). However it is not a gold-standard diagnostic test as sensitivity can be low (28.5%) in early stages of infection (135) and can only be used in cattle post-mortem.

In Cameroon, the use of ante-mortem diagnostics has been limited to sporadic field studies (117; 77; 366; 114; 75; 120; 116; 115; 118). Most ante-mortem bTB diagnostics are based upon measuring specific aspects of the immune response to *M. bovis* antigens (193; 48; 78). Like other infectious diseases detection of a humoral response to *M. bovis* is possible by ELISA or immuno-diffusion testing of serum (258; 187). However false negatives are common as humoral responses are variable between individual cattle and mainly associated with chronic stages of infection (256). Cell-mediated immune responses are thought to predominate in the majority of *M. bovis* infections and subsequently diagnostics based on these responses are

commonly used in describing bTB epidemiology (256; 140; 264). The principle of CMI diagnostic tests is that *M. bovis* infected cattle produce a CMI response when exposed to a cocktail of *M. bovis* PPD (193). Cell-mediated immunity based diagnostic tests include the in-vivo tuberculin skin tests (SIT and SCITT) and the in-vitro IFN-γ assay. The IFN-γ assay has been of interest for describing bTB epidemiology, as it is reported to identify early infections from 1-4 weeks post infection that often precede detection by skin test responses or lesion development (78). Subsequently the IFN-γ assay has a reported higher sensitivity (73-100%) compared to the skin tests with a reasonable specificity (85.0-99.6%). A test with a high sensitivity would be advantageous in estimating prevalence of a disease that has zoonotic consequences in a setting with no control measures (167; 85; 367) to minimise false negative results. Furthermore the assay can be used in the field without the need for a return visit, unlike the skin tests, so be could valuable in epidemiological surveys in rural Cameroon.

However the performance of the IFN-γ assay varies between different cattle populations and can misclassify animals as false positives or negatives (163; 368; 124; 369). Similarly the performance of the IFN-γ assay in Cameroon will impact on accuracy of bTB prevalence estimated. Identifying the factors that lead to false positive and false negative results might allow the performance of the IFN-γ assay to be improved by controlling for these factors (370; 371; 85). Environmental factors such as presence of other mycobacteria can increase the number of false positives (372; 166). Host factors such as breed-specific differences in immune responses to *M. bovis* (373) and immunosuppression of the individuals immune response by co-infections such as *Fasciola spp* have been shown to lead to false negative results (298). As the IFN-γ assay is potentially useful for describing bTB epidemiology in Cameroon, it would be useful to know which factors, specific to this

setting, influence animals being misclassified by the test.

In the absence of a gold-standard diagnostic for bTB, an abattoir study was conducted to compare the agreement of the IFN-γ assay with pathological and serological responses at post-mortem. The first aim of this chapter is to describe the sample from the population of cattle slaughtered in Bamenda and Ngaoundere abattoirs in Cameroon. The performance of the IFN-γ assay is investigated compared to TB lesion identification at PME and *M.bovis* serology. A positive cut-off value is selected for the IFN-γ assay to be used to identify bTB positive cattle in this setting. The the estimated prevalence of bTB in slaughtered cattle in Cameroon is described. Finally the reasons for IFN-γ assay false positive and negative cattle are explored.

4.2 Materials and methods

4.2.1 Abattoir study and bovine tuberculosis diagnostic tests

The abattoir study dataset is only considered in this chapter with the study design/ sampling methodology are described in detail in section 3.2.4. A total of 2064 cattle were sampled in the abattoir study. A combination of bTB diagnostic tests were performed in the abattoir study which will be included in the analysis of this chapter:

1. IFN-γ assay (Bovigam®) (n=1979)
2. Bovine tuberculosis lesion identification at meat inspection (Meat inspection; n=2064).
3. *M.bovis* serology (IDEXX® *M.bovis* ELISA) (n=2001).

Not all cattle were tested by the 3 diagnostic tests due to logistics of sampling in municipal abattoirs. Where subsets of the abattoir dataset are used in the analysis, only animals with complete sets of variables are used. Denominators of subsets are stated in the associated text. Diagnostic test methodology and interpretation formulae are described in section 3.3.

4.2.2 Statistical analyses

Statistical analyses are performed using packages and functions in R (343). Graphics are produced using the *ggplot2* package (344). Maps are drawn using QGIS 2.2® (345) and shape files obtained from the open access GADM database of Global Administrative Areas (www.gadm.org). The animal and TB lesions samples are described using proportions and exact binomial 95% CIs for lesion prevalence

estimates to compare differences between the two abattoir samples (348). Estimated values are calculated using the *svyby* of the *survey* package (346). Re-categorisation of the DS and BCS variables is described in the results section of this chapter to be used in IFN-γ false positive and negative analysis (Multivariable logistic regression (MLR) models).

To summarise TB lesion severity an overall lesion score (OLS) was created to combine the four TB lesion scores (Pathology, type, size and scale scores) for each bovine (Explained in section 3.3.4). The calculated OLS was then used to investigate the correlation between TB lesion severity and IFN-γ response. Cattle with no lesions were given an OLS score of 0 for each of the four TB lesion scores. Subsequently principle component analysis (PCA) was used to calculate OLS using lesion positive animals only (Section 3.5.1). Where bovines had multiple lesions, an arthemetic mean of the OLS for each TB lesion was taken. The OLS was used to measure variation in extent of TB lesions and produce a single score per animal. The *princomp* function in the *stats* package (343) was used to calculate OLS and PCA plots were created using *ggbiplot* function in the *ggplot2* package to demonstrate the variance explained by the summary variable (350). The PCA calculated four principle components (PCs) to explain the variance between the four TB lesion scores. Subsequently the PC which explains the most variance, of the 4 TB lesion scores, was selected to create an OLS, converted to a positive value and +4 added to create an appropriate positive numeric scale for further analysis.

Diagnostic agreement was investigated for the IFN-γ assay, TB lesion identification at meat inspection and *M. bovis* serology stratified by abattoir. Firstly agreement of raw diagnostic test results was investigated using scatter plots comparing two tests at a time (The equations for calculation of raw diagnostic results are included in section 3.3). Including:

1. Numeric IFN-γ difference in OD for the IFN-γ assay.
2. Binary positive and negative result for TB lesion identification at meat inspection.
3. Numeric S/P ratio or binary positive and negative result for *M. bovis* antibody ELISA (*M. bovis* serology).

Spearman's correlation coefficient (r) was used to determine whether a correlation between OLS and numeric diagnostic test results is significant using the significance level of $p<0.05$, using the *cor* function in the *stats* package (343). Percentage agreement and Cohens kappa statistic were used to quantify agreement between test pairs. The functions used to calculated these values were the *agree*, *kappa2* and *rater.bias* in the *irr* package (359). Cohens kappa statistic was interpreted as =1 (perfect agreement), 0.81-1 (almost perfect agreement), 0.61-0.8 (substantial agreement), 0.41-0.6 (moderate agreement), 0.21-0.4 (fair agreement), 0.01-0.2 (poor agreement), $\leq$0 (No agreement) (352).

The sensitivity and specificity were calculated for the IFN-γ assay to estimate the tests diagnostic performance (Section 3.5.2) relative to TB lesion identification. There is no gold-standard diagnostic test for bTB, thus IFN-γ assay false positives and negatives identified are relative to the specificity and sensitivity of the diagnostic test compared to (e.g. TB lesion identification at meat inspection). These relative false positives and negatives are subsequently referred to as "false positives or negatives" for ease. Factors which influence false positive and negative IFN-γ assay results, were investigated by comparison to TB lesion status by abattoir. MLR models were used to investigate the two forms of IFN-γ assay and lesion binary disagreement:

1. **IFN-γ assay false positives:** IFN-γ assay positive and TB lesion negative.
2. **IFN-γ assay false negatives:** IFN-γ assay negative and TB lesion positive.

For each abattoir, reasons for false positive and negative IFN-γ assay results were investigated. Two subsets of the abattoir data were used to investigate false positive (Figure 4.1) and negative (Figure 4.2) IFN-γ assay results. Hence using MLR analysis 4 MLR models were produced for each abattoir, to investigate reasons for false positive (2 MLR models) and negative (2 MLR models) results. For each abattoir the data was subsetted, to be used in the four MLR models, by the four diagnostic outcomes (IFN-γ assay positive or negative and TB lesion positive or negative). Then the outcome variable of each of the 4 MLR models was the binary result of contrary diagnostic test (e.g. subset positive IFN-γ assay and outcome variable TB lesion negative).

Intrinsic animal level variables were included as explanatory variables in MLR model selection, if they were considered to be potentially biologically significant to development of TB lesions and/ or IFN-γ responses to *M. bovis*. The dentition score (DS) variable was recategorised to <3 years (DS<2) and $\geq$3 years (DS$\geq$2) as a proxy for cattle age. The breed variable was recategorised to mixed breed (Mixed breed and Gudali), Exotic (*B. taurus* cattle) and Fulani breed (Red and White Fulani) due to small numbers of cattle in some subsets (Red Fulani and Gudali breeds). Culture results were recategorised into negative (Negative on all media culture), *M. bovis* positive (MTC positive on one or more of the three media types) and NTM positive (NTM positive on one or more of the three media types). The MLR models were constructed using the *glm* function in the *stats* package (343). Model selection was based on the Akaike information criterion (AIC) and the final best model was selected using the lowest AIC (Described further in section 3.5.3). All relevant explanatory variables included in MLR models, had their interactions investigated during model selection (360). The final model selected was verified by computing ΔAIC using the package *AICcmodavg* and *modavg* function (363). The p value, odds ratio with 95% CI of each explanatory variable in the final MLR model were also reported.

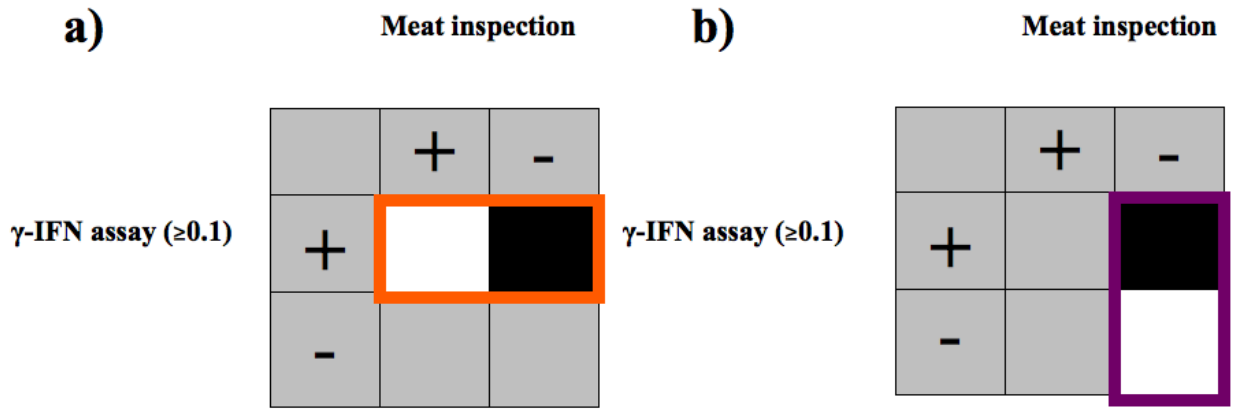


Figure 4.1: **Diagrammatic representation of the subsetting of the data to investigate risk factors for IFN-gamma assay false positives.** Model a) Subset of all cattle with a positive IFN-γ assay response highlighted in orange (n=120) with TB lesion as the dependent variable. Model b) Subset of all cattle negative for TB lesions highlighted in purple (n=1834) with IFN-γ assay as the dependent variable. Black areas indicates "positive" (Disparate results) and white areas indicates "negative" (Agreeing results) of the dependent variable.

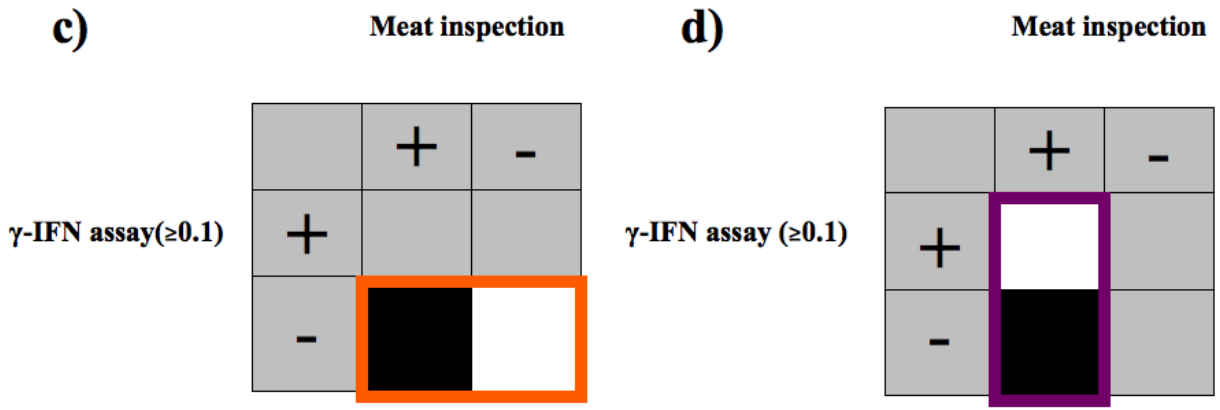


Figure 4.2: **Diagrammatic representation of the subsetting of the data to investigate risk factors for IFN-gamma assay false negatives.** Model c) Subset of all cattle with a negative IFN-γ assay response highlighted in orange (n=1744) with TB lesion as the dependent variable. Model d) Subset of all cattle positive for TB lesions highlighted in purple (n=139) with IFN-γ assay as the dependent variable. Black areas indicates "positive" (Disparate results) and white areas indicates (Agreeing results) of the dependent variable.

4.3 Results

4.3.1 Cattle sample

Of the total 2064 slaughtered cattle that were sampled; 1129 are from Bamenda abattoir and 935 from Ngaoundere abattoir. Not all animals slaughtered in Ngaoundere abattoir originated from the VD (Figure 4.3) with 0.1% from other Adamawa Region divisions and 31.5% from the North Region (Appendix H). Younger male cattle (<3 years) were predominantly slaughtered at Bamenda abattoir and older female cattle ($\geq$3 years) in Ngaoundere abattoir (Figure 4.4). The majority of slaughtered cattle sampled in Bamenda abattoir (NWR) were Fulani breed while in Ngaoundere abattoir (VD) the majority are mixed breed (Table 4.1).

All cattle were meat inspected for TB lesions at slaughter as part of routine meat inspection. Due to logistical problems during sampling in the abattoirs, only 1105/1129 cattle in Bamenda abattoir and 874/935 cattle in Ngaoundere abattoir had IFN-γ assay results. Furthermore 1126/1129 cattle sampled in Bamenda abattoir and 875/935 cattle sampled in the Ngaoundere abattoir had *M. bovis* serology results. Additionally 1115/1129 cattle sampled in Bamenda abattoir and 821/935 sampled in Ngaoundere abattoir were inspected for evidence of *Fasciola* pathology. For the remainder of the results when diagnostic test results are compared, only analysis includes cattle with both diagnostic test results (denominators are stated in the associated text).

4.3.2 TB lesion sample

In total 7.3% cattle (n=2064, CI: 6.2-8.4%) had TB lesions identified at slaughter (Bamenda and Ngaoundere). In total 270 lesions (Bamenda n=84; Ngaoundere

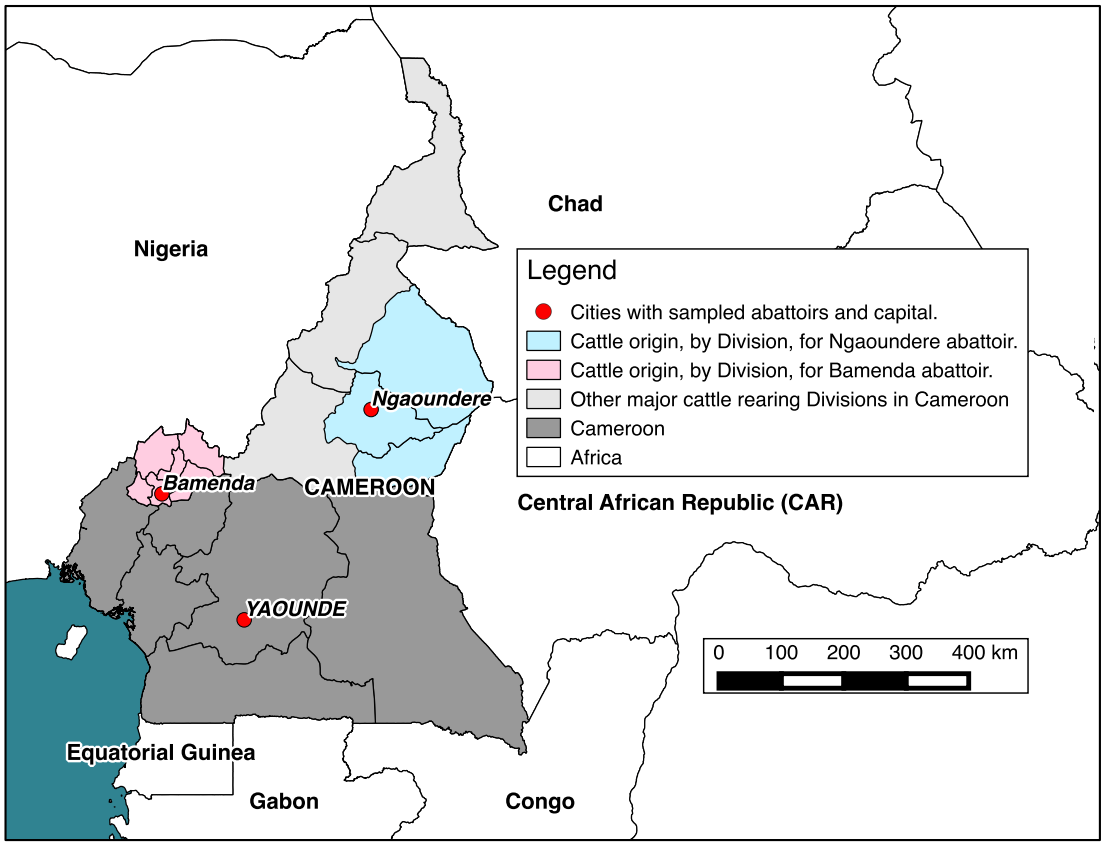


Figure 4.3: **Map of Cameroon showing where sampled slaughtered cattle originated.** The pink area shows the cattle sampled from Bamenda abattoir originated from all 7 Divisions of the NWR only. Cattle sampled in Ngaoundere abattoir (Blue) originated from the VD and Mbere Division of the Adamawa Region along with the Mayo Rey Division of the North Region.

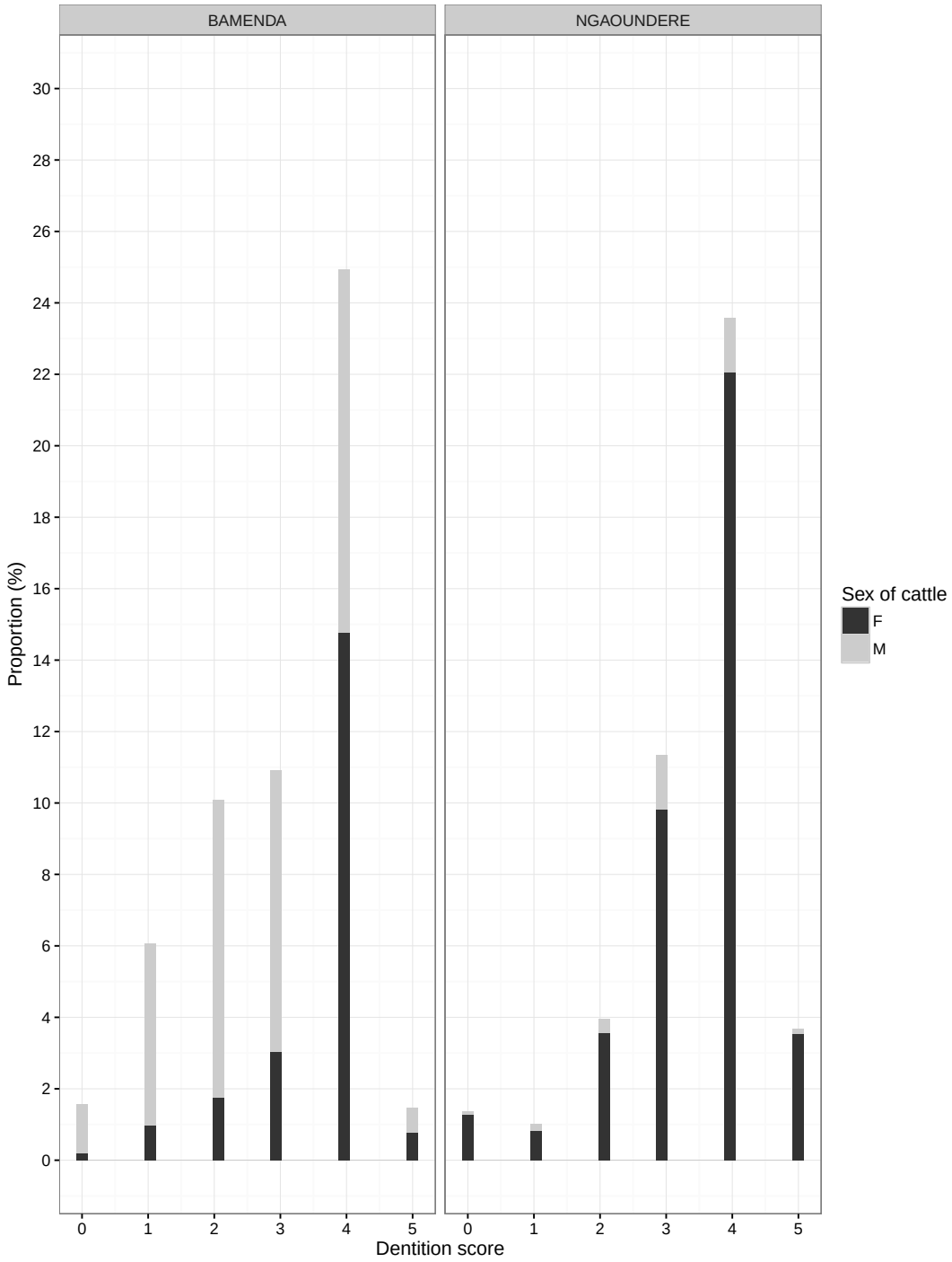


Figure 4.4: **The distribution of sampled cattle by sex, age and abattoir.** Proportions by abattoir are the proportions of total number of cattle sampled, subsetted per abattoir, with a full set of data (Bamenda n=1129; Ngaoundere n=935). Dentition score is re-catagoried for the remainder of this thesis to age <3 years (0-2) and $\geq$3 years (3-5). Sex of animal: F= Female; M= Male.

	Bamenda abattoir (n=1129)	Ngaoundere abattoir (n=935)
Sex		
Female	39.0% (440/1129)	91.2% (853/935)
Male	61.0% (689/1129)	8.5% (79/935)
Unknown	0.0% (0/1129)	0.3% (3/935)
Age (By dentition score (DS))		
< 3 years (0-2)	32.1% (362/1129)	13.9% (130/935)
>= 3 years (2-5)	67.6% (763/1129)	84.8% (793/935)
Unknown	0.3% (4/1129)	1.3% (12/935)
Breed		
Mixed breed	15.1% (170/1129)	68.7% (642/935)
Fulani	84.5% (954/1129)	30.8% (288/935)
Exotic	0.4% (5/1129)	0.0% (0/935)
Unknown	0.0% (0/1129)	0.5% (5/935)
Body condition score (BCS)		
Thin (1-2)	25.8% (291/1129)	21.8%(204/935)
Normal (3)	63.8% (720/1129)	64.4% (602/935)
Fat (4-5)	10.2% (115/1129)	13.3% (124/935)
Unknown	0.2% (3/1129)	0.5% (5/935)

Table 4.1: **Descriptive summary of slaughtered cattle from the abattoir study, subsetted by Bamenda and Ngaoundere abattoirs (n=2064).**

n=186) were examined from 151 cattle (Bamenda n=45; Ngaoundere n=106) with half of TB lesioned cattle having more than one TB lesion (Table 4.2). There were much fewer TB lesions identified in tissues (7.5%, CI: 4.1-10.9%) than in LNs (92.5%. CI: 89.1-95.9%). More TB lesions were identified in Ngaoundere abattoir (11.3% CI: 9.3-13.4%) than Bamenda abattoir (4.0% CI: 2.8-5.1%). Predominately TB lesions were recovered from retropharyngeal (Bamenda 36.9%, CI: 27.4-47.6% and Ngaoundere 31.2%, CI: 25.0-38.2%), bronchial (Bamenda 23.8%, CI:15.9-34.0% and Ngaoundere 16.7%, CI: 12.0-22.7%) and mediastinal LNs (Bamenda 15.5%, CI: 9.2-24.8% and Ngaoundere 18.8%, CI: 13.8-25.1%) with no difference between abattoirs. More TB lesions from other LNs were identified in Bamenda (14.3%, CI: 8.3-23.4%) than Ngaoundere (3.8%, CI: 1.8-7.7%) abattoir, including mandibular, prescapular and prefemoral. A larger proportion of TB lesions were recovered from livers of cattle in Ngaoundere (11.8%, CI: 7.9-17.3%) compared to none in Bamenda (0.0%, CI: 0.0-4.3%) (Figure 4.5).

All TB lesions were graded by pathology, type, size and scale (Figure 4.6). In Ngaoundere there were less scale 4 lesions (0% CI: 0.0-2.0%) than $\leq$ scale 3 lesions. There were also fewer scale 4 lesion in Ngaoundere than in Bamenda (29.6% CI: 20.7-40.4%). The type score demonstrated a similar relationship with more type 2 (caseous) lesions identified in both abattoirs than type 1 (mucoid/ purulent) and 3 (calcified). TB lesion size differed between the two abattoirs with much smaller TB lesions (size score 1 (<10mm); 64.2% CI: 53.3-73.8%) identified in Bamenda abattoir than Ngaoundere (size score 3 (50mm); 49.4% CI: 41.7-57.1%). In Ngaoundere cattle were found to have singular TB lesions (scale score 1; 84.2% CI: 77.6-89.1%) compared to Bamenda where singular and multiple tuberculous presentations occur (scale score 1 (55.6 CI: 44.7-65.9%) and 2 (44.4% CI: 34.1-55.3%) respectively).

To summarise information about the extent of the severity of TB lesion pathology an

overall lesion score (OLS) was calculated using the 4 different types of TB lesion grading using PCA analysis (Section 3.5.1). Principle component 1 (PC1) explained the majority of the variance of the four lesion scores (63.8%). Both PC1 and PC2, the next component that explained the most variation (18.0%), were compared (Figure 4.7) to see if OLS differed between abattoirs. PC1 and PC2 summarised the variation in the 4 TB lesion variables for both abattoirs (Two larger circles do overlap). PCA1 was converted to become the OLS, as described in subsection 4.2.2. Using OLS of TB lesion positive cattle (n=151) no statistical difference was noted in the TB lesion severity between the two abattoirs (Bamenda mean OLS:4.22; CI: 4.01-4.43. Ngaoundere mean OLS: 3.90; CI: 3.62-4.19) abattoir (p=0.72) although prevalence of TB lesions differed between the two abattoirs.

All 270 TB lesions were cultured for mycobacteria and the majority of TB lesions were culture positive (Bamenda 83.3%, CI: 73.9-89.8% and Ngaoundere 82.7%, CI: 76.4-87.5%). Of these 68.9% were culture positive for *M. bovis* from Bamenda (CI: 55.2-82.6%) and 65.0% from Ngaoundere (CI: 56.0-74.2%). For non-tuberculous mycobacteria (NTM) 6.7% (CI: 1.4-18.3%) and 6.6% (CI: 2.7-13.1%) were culture positive from Bamenda and Ngaoundere respectively.

A small randomly selected subset of TB lesion negative cattle (n=179) had a retropharyngeal LN cultured. These were sampled using a random number table to sample 2-3 cattle that did not have TB lesions at meat inspection. Only 1.7% (CI: 0.3-4.8%) are *M. bovis* culture positive and 11.7% are NTM (CI: 7.9-17.8%) culture positive.

Number of lesions identified at meat inspection	Bamenda abattoir n=45 (95% CI)	Ngaoundere abattoir n=106 (95% CI)
1	57.0% (43.2-72.4%)	55.7% (46.2-65.2%)
2	22.2% (9.9-34.5%)	21.7% (13.8-29.6%)
3 or more	20.0% (8.2-31.8%)	22.6% (14.6-30.6%)

Table 4.2: **Frequency of lesions reported in lesioned cattle in the abattoir study (Bamenda n=84, Ngaoundere n=186).**

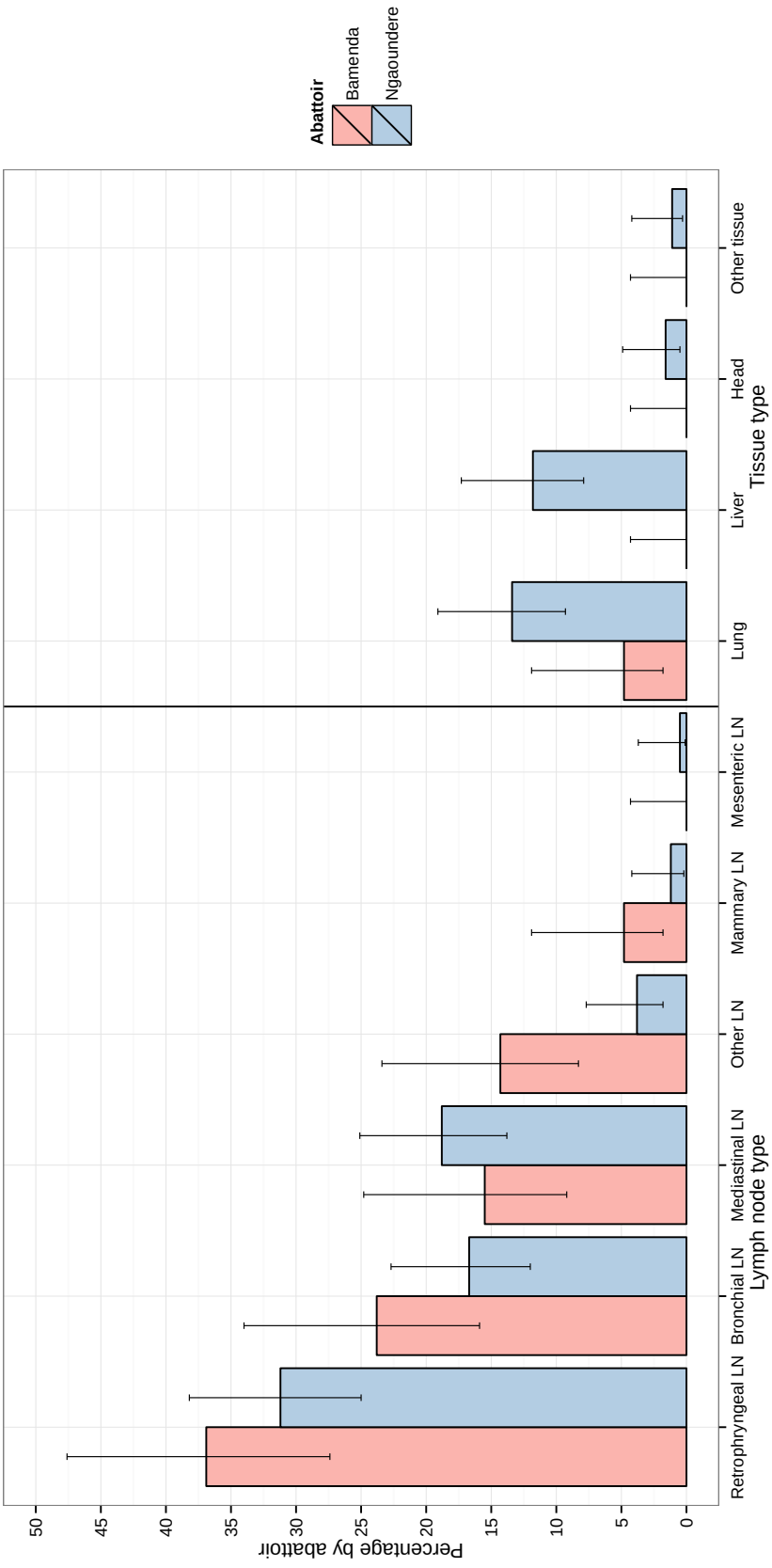


Figure 4.5: **Proportion of TB lesions identified in each tissue type by abattoir (Bamenda n=84, Ngaoundere n=186).** Proportions are total number of lesions per abattoir (n=270).

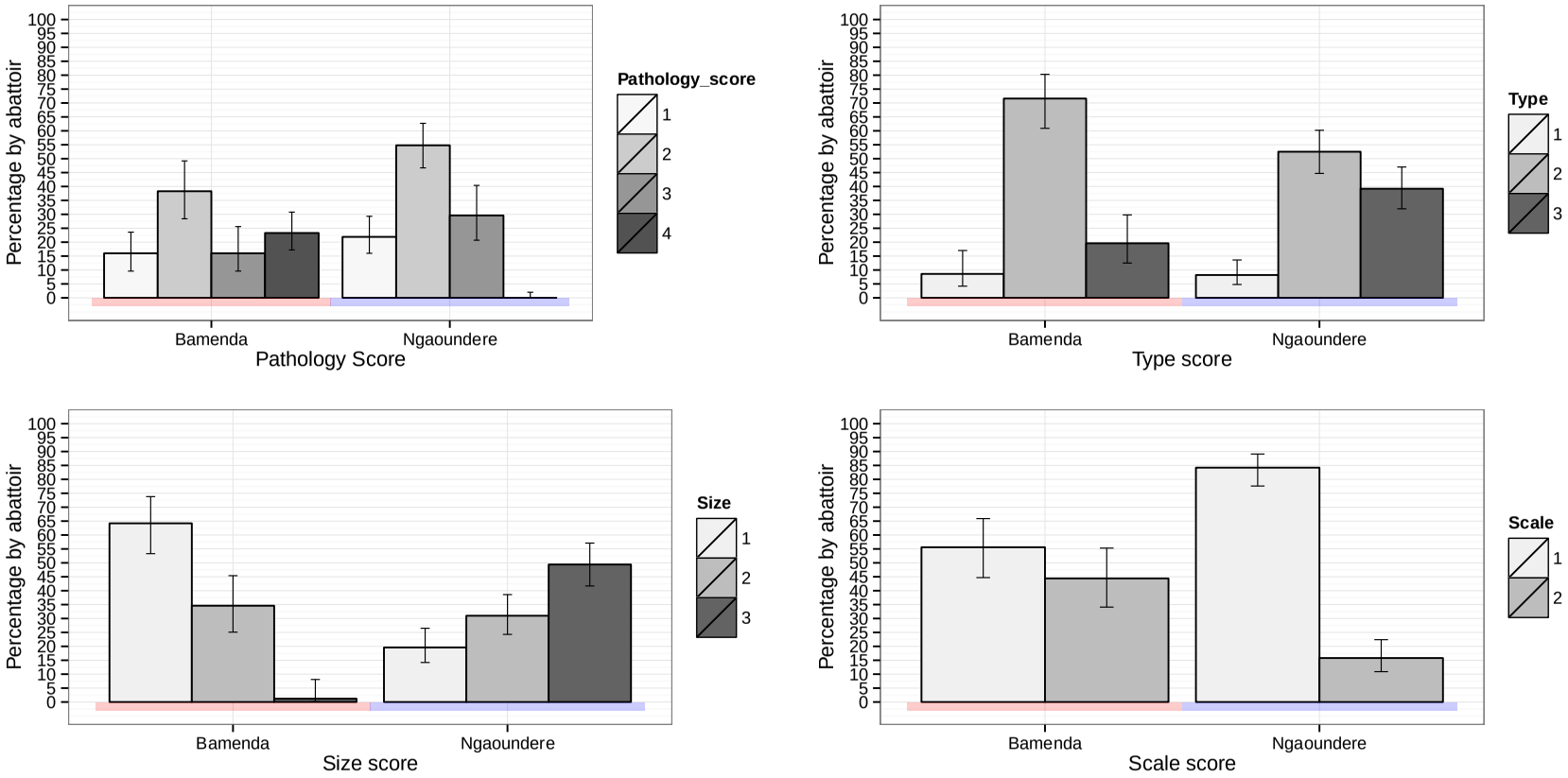


Figure 4.6: **Proportion of TB lesions with pathology, type, size and scale scores (Bamenda n=84, Ngaoundere n=186).** Proportions by abattoir are the proportions of total number of lesions (n=270).

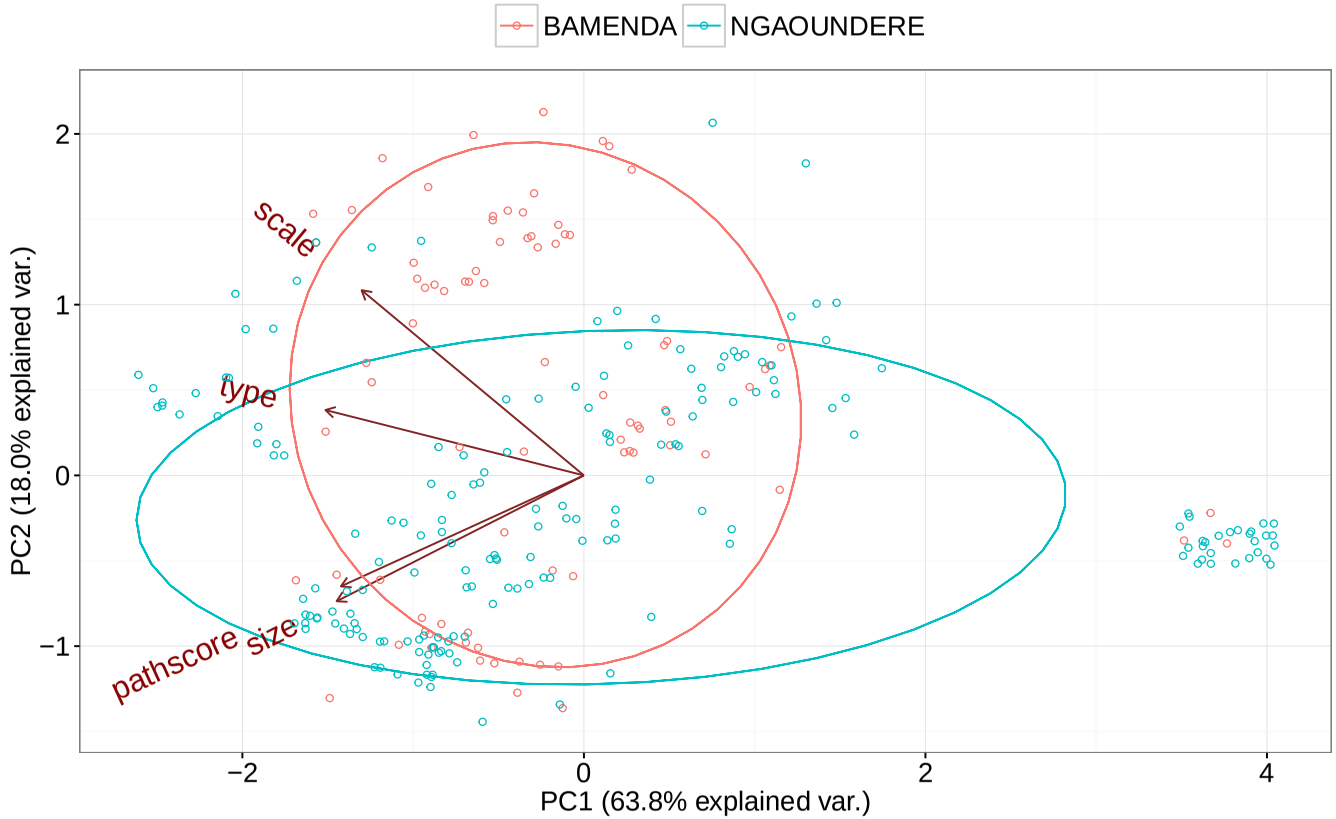


Figure 4.7: **Comparing principle component 1 (PC1) to principle component 2 (PC2) from PCA for summerising the variation in the 4 lesion scores (270 from 151 lesion positive cattle).**

PC1 explains the most variation (63.4%) and PC2 explains the second most (18.0%). The four lesion scores are Pathology (0=No visible lesion; 1=No gross lesion but visible on slicing; 2=Less than or equal to 5 gross lesions; 3=Gross coalescing lesion), Type (0=No visible lesion; 1=Mucoid/ purulent; 2=Caseous; 3=Calcified), Size (0=No visible lesion; 1=Less than 10mm; 2=10-50mm; 3=Greater than 50mm) and Scale (0=No visible lesion; 1=Single; 2=Multiple). Points are coloured from Bamenda (Red) and Ngaoundere (Blue) abattoirs. Arrows demonstrate loading of the four variables. The larger circles denote where the majority of the lesions lie per abattoir. A small cluster of 37 lesions (From 8 lesion positive cattle) with low lesion grades (On all four grades) are noted on the right of the plot.

4.3.3 Comparison of bovine tuberculosis diagnostic test cut-off values

IFN-gamma assay and TB lesion identification at meat inspection

The results of the IFN-γ assay are plotted by abattoir and TB lesion status in figure 4.8, with the recommended cut off values marked ($\geq$0.05 and $\geq$0.1 positive cut-off values). In both abattoirs the majority of cattle tested appear to be IFN-γ assay ($\geq$0.05 and $\geq$0.1 positive cut-off value) and TB lesion negative. The pattern of agreement and disagreement between TB lesion identification and the IFN-γ assay appears to be similar for Bamenda and Ngaoundere. To quantify the agreement between the two tests, agreement statistics are calculated for both abattoirs separately (Tables 4.3 and 4.4). Percentage agreement is highest (Bamenda= 93.7%; Ngaoundere= 87.9%) for IFN-γ assay using $\geq$0.1 (Tables 4.3 and 4.4). Furthermore the Cohens kappa statistic suggests that overall best agreement between the IFN-γ assay ($\geq$0.1) and TB lesion status was "fair" (κ= 0.21-0.4) in both abattoirs. Subsequently the $\geq$0.1 positive cut-off value for the IFN-γ assay will be used for the rest of the analysis in this thesis, due to having the best agreement in both abattoirs. Although TB lesion identification is not a gold-standard diagnostic test for bTB it was used to calculate a comparative sensitivity and specificity for the IFN-γ assay ($\geq$0.1) to provide an estimation of diagnostic performance in Cameroon. Although sensitivity and specificity was similar in both abattoirs, specificity was much higher (Bamenda= 95.7% CI: 94.3-96.8%; Ngaoundere= 94.8% CI: 93.0-96.3%) than sensitivity (Bamenda= 45.5% CI: 30.4-61.2%; Ngaoundere= 34.65% CI: 25.5-44.7%) in both abattoirs.

There was no correlation between between OLS and IFN-γ responses (Bamenda r=0.12; Ngaoundere r=0.09) implying that severity of TB lesion is not correlated with IFN-γ responses.

Using at test cut-off of $\geq$0.1 the proportion of disagreement for TB lesion status negative/ IFN-γ assay positive cattle (False positives) was similar in Ngaoundere (4.3% CI: 3.0-5.6%) and Bamenda (4.1% CI: 2.9-5.2%). The proportion of TB lesion positive/ IFN-γ assay negative cattle (false negatives) was higher in Ngaoundere (7.6% CI: 5.9-9.3%) than Bamenda (2.2% CI: 1.4-3.1%).

M. bovis serology, IFN-gamma assay and TB lesion identification at meat inspection

Firstly, using $\geq$0.05 and $\geq$0.1 positive cut-off values, the results for the IFN-γ assay were dichotomised and compared to the *M. bovis* serology result by abattoir (Figure 4.9). For both abattoirs, agreement between IFN-γ assay and *M. bovis* serology negative results (Area shaded yellow) appears to be consistent. A small proportion of cattle had negative *M. bovis* serology and positive IFN-γ assay results in both abattoirs (using $\geq$0.1 Bamenda= 2.1%, CI: 1.3-3.1%; Ngaoundere= 5.5%, CI: 4.1-7.2%). Agreement appears to be poor between *M. bovis* serology positive and IFN-γ assay results. Particularly in Bamenda where a large proportion of the sample disagreed with a positive bTB serology and negative IFN-γ assay (Using $\geq$0.1 Bamenda= 46.7%, CI: 43.8-49.7%; Ngaoundere= 9.3%, CI: 7.4-11.4%). Focusing on Bamenda abattoir percentage agreement with IFN-γ assay and *M. bovis* serology, at either cut-off, was low ($\sim$50%) and the Cohens kappa statistic suggested "poor" agreement (κ= 0.01-0.2) (Table 4.3). For Ngaoundere abattoir the Cohens kappa statistic suggested "poor-fair" considering the CIs (Table 4.4).

Secondly, the TB lesion status (Positive or negative) was compared to the numeric *M. bovis* antibody ELISA S/P ratio by abattoir (Figure 4.10). Agreement appeared to be poor particularly for Bamenda abattoir (Bamenda= 50.6%; Ngaoundere= 83.2%). In

Bamenda agreement between binary *M. bovis* serology and TB lesion status was "poor" (Table 4.3) and "poor-fair" considering the CI for Ngaoundere (Table 4.4). Additionally no correlation was noted between *M. bovis* serology and OLS suggesting that severity of lesion is not correlated with *M. bovis* serology (Bamenda r=0.03; Ngaoundere r=0.10).

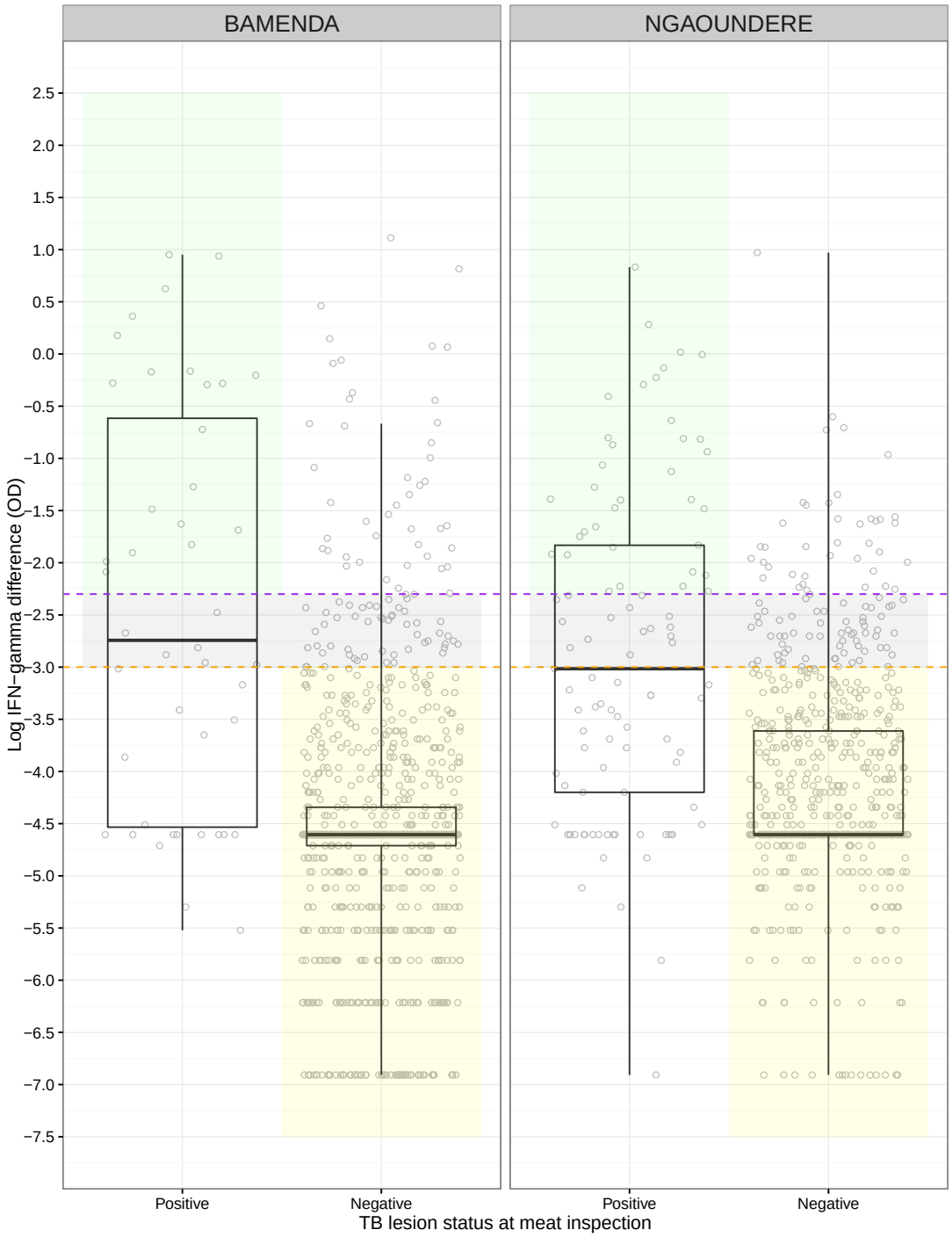


Figure 4.8: **Box plot of the log IFN-gamma assay raw difference between avian and bovine reactions and TB lesion status for slaughtered cattle by abattoir (Bamenda n=1105, Ngaoundere n=874).**

Points are jittered to avoid overlap. For the IFN-γ assay $\geq$0.05 (Orange line) and $\geq$0.1 (Purple line) positive cut-off values are shown. For TB lesion status, positive and negative results are shown. The green area denotes the test positive cattle for the IFN-γ assay ($\geq$0.1) and positive for meat inspection. The grey area denotes proportion of additional test positive cattle for the IFN-γ assay ($\geq$0.05). The yellow area denotes test negative cattle for IFN-γ assay (<0.05) and TB lesion status.

Bamenda abattoir (n=1129)					
IFN-gamma assay	**TB lesion identification (n=1105)**		**Percentage agreement**	**Cohens kappa statistic**	**Cohens kappa statistic 95% CI**
>=0.05		+	-	89.8%	0.27	0.18-0.36
	+	26	95			
	-	18	966			
>=0.1		+	-	93.7%	0.33	0.21-0.45
	+	20	46			
	-	24	1015			
IFN-gamma assay	***M. bovis* serology (n=1104)**		**Percentage agreement**	**Cohens kappa statistic**	**Cohens kappa statistic 95% CI**
>=0.05		+	-	51.1%	0.03	0.01-0.07
	+	70	51			
	-	489	494			
>=0.1		+	-	51.2%	0.03	0.01-0.06
	+	43	23			
	-	516	522			
TB lesion identification	***M. bovis* serology (n=1126)**		**Percentage agreement**	**Cohens kappa statistic**	**Cohens kappa statistic 95% CI**
		+	-	50.6%	0.03	0.01-0.05
	+	30	14			
	-	542	540			

Table 4.3: **Comparisons of agreement and Cohens kappa statistic between IFN-gamma assay ($\geq$0.05 and $\geq$0.1), TB lesion identification at meat inspection and *M. bovis* serology for slaughtered cattle in Bamenda abattoir.**

Ngaoundere abattoir (n=935)					
IFN-gamma assay	**TB lesion identification (n=874)**		**Percentage agreement**	**Cohens kappa statistic**	**Cohens kappa statistic 95% CI**
		+	-			
>=0.05	+	50	102	82.5%	0.30	0.21-0.38
	-	51	671			
		+	-			
>=0.1	+	35	40	87.9%	0.33	0.23-0.43
	-	66	733			
IFN-gamma assay	***M. bovis* serology (n=872)**		**Percentage agreement**	**Cohens kappa statistic**	**Cohens kappa statistic 95% CI**
		+	-			
>=0.05	+	40	112	79.4%	0.19	0.11-0.27
	-	68	652			
		+	-			
>=0.1	+	27	48	85.2%	0.22	0.12-0.31
	-	81	716			
	***M. bovis* serology (n=875)**		**Percentage agreement**	**Cohens kappa statistic**	**Cohens kappa statistic 95% CI**
TB lesion identification		+	-			
	+	31	70	83.2%	0.20	0.11-0.29
	-	77	697			

Table 4.4: **Comparisons of agreement and Cohens kappa statistic between IFN-gamma assay ($\geq$0.05 and $\geq$0.1), TB lesion identification at meat inspection and *M. bovis* serology for slaughtered cattle in Ngaoundere abattoir.**

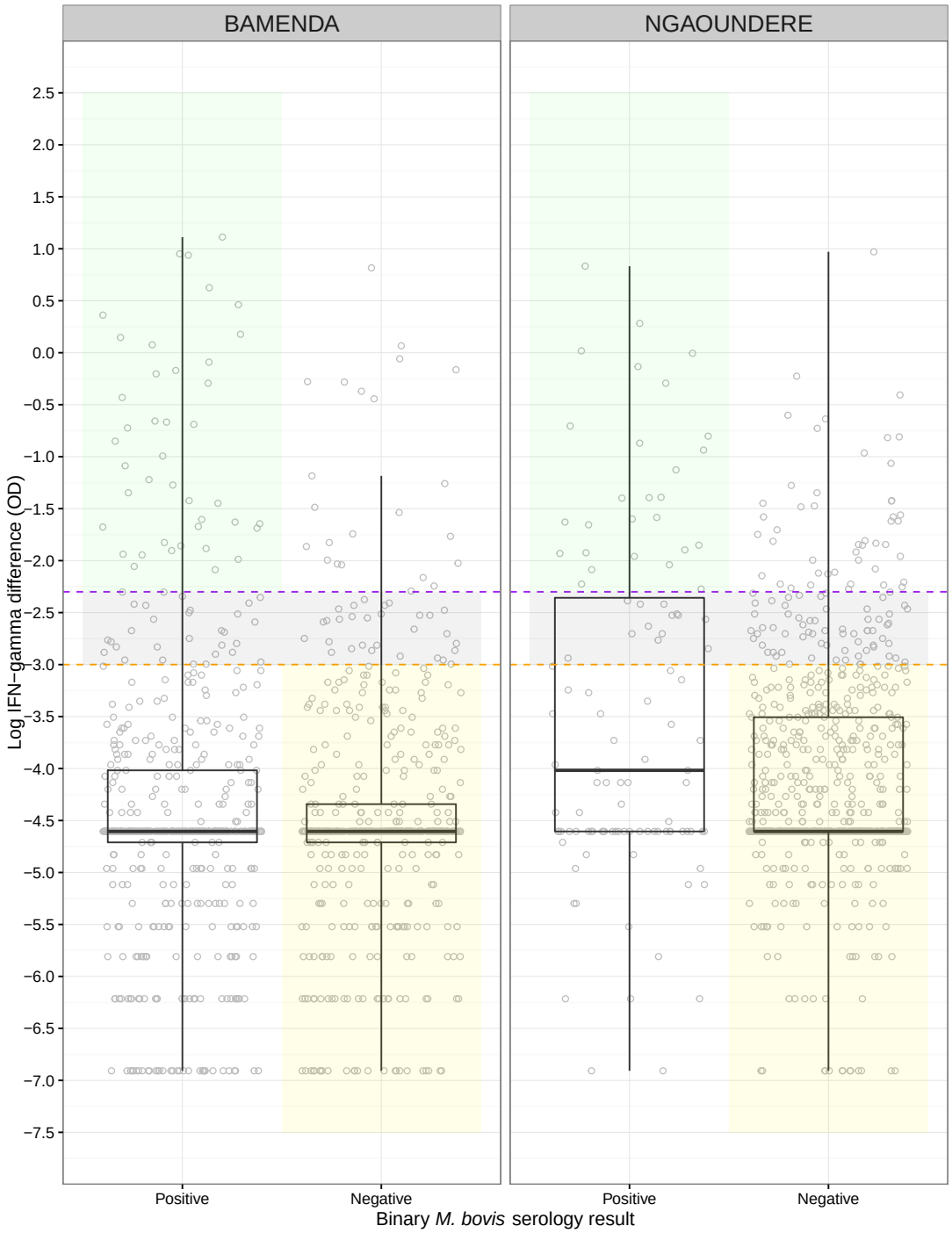


Figure 4.9: **Box plot of the log IFN-gamma assay raw difference between avian and bovine reactions and *M. bovis* serology status for slaughtered cattle by abattoir (Bamenda n=1104, Ngaoundere n=872).**

Points are jittered to avoid overlap. For the IFN-γ assay $\geq$0.05 (Orange line) and $\geq$0.1 (Purple line) positive cut-off values are shown. For the *M. bovis* serology, positive and negative results are shown. The green area denotes the test positive cattle for the IFN-γ assay ($\geq$0.1) and positive for *M. bovis* serology. The grey area denotes proportion of additional test positive cattle for the IFN-γ assay ($\geq$0.05). The yellow area denotes test negative cattle for IFN-γ assay (<0.05) and *M. bovis* serology status.

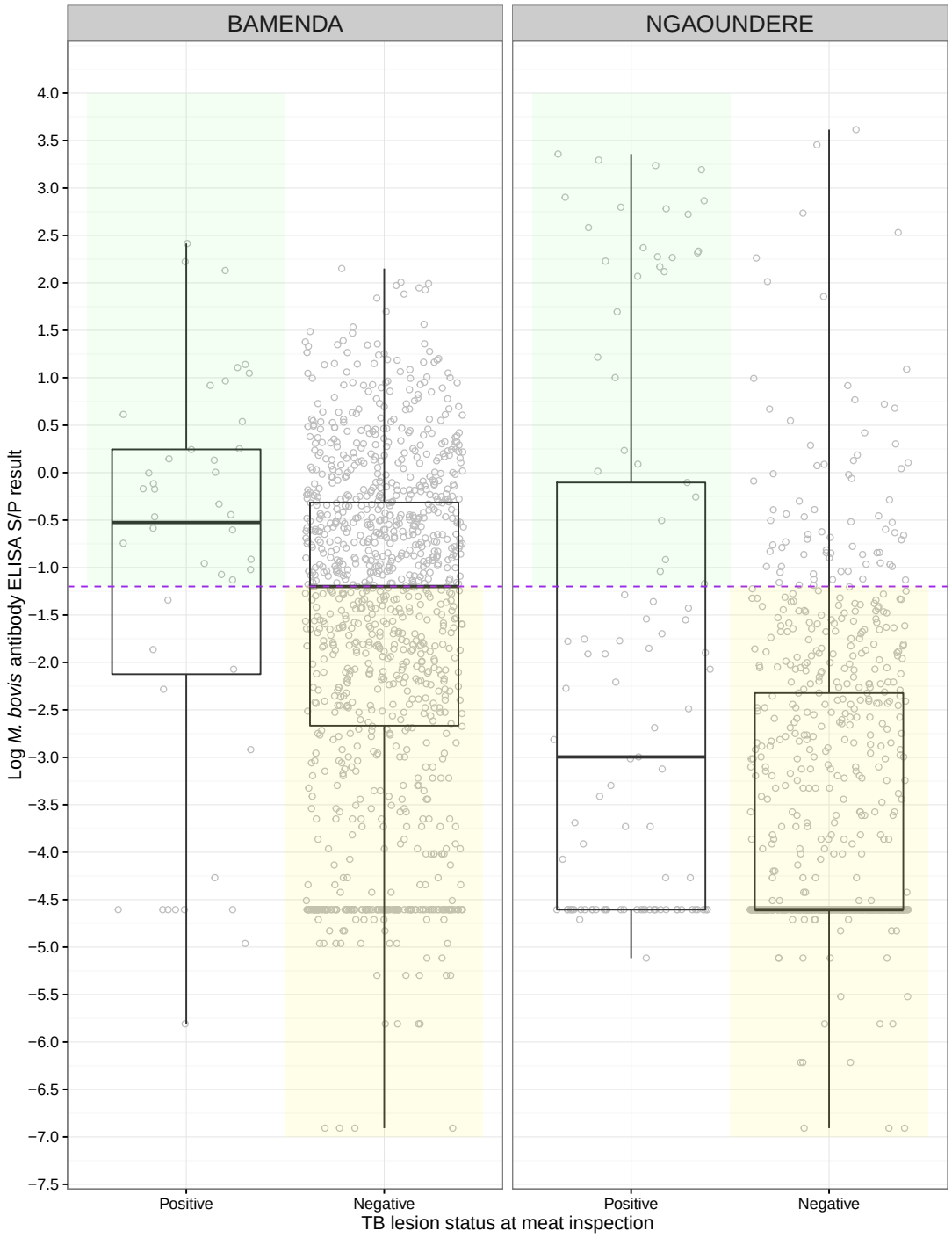


Figure 4.10: **Box plot of the log *M. bovis* antibody ELISA S/P result (*M. bovis* serology) and TB lesion status for slaughtered cattle by abattoir (Bamenda n=1126, Ngaoundere n=875).**

Points are jittered to avoid overlap. For the *M. bovis* antibody ELISA ≥0.3 (Purple line) positive cut-off values are shown. For TB lesion status, positive and negative results are shown. The green area denotes the test positive cattle for the *M. bovis* antibody ELISA (≥0.3) and positive for TB lesion. The yellow area denotes test negative cattle for the *M. bovis* antibody ELISA (<0.3) and TB lesion status.

4.3.4 Proportion of cattle TB lesion, IFN-gamma assay and *Fasciola* pathology positive

The proportion of cattle IFN-γ assay ($\geq$0.1), TB lesion and *Fasciola* pathology positive was calculated for each abattoir (Table 4.5). Overall there was no difference in the proportion of IFN-γ positive cattle between Bamenda and Ngaoundere abattoirs. More Fulani cattle are IFN-γ positive in Ngaoundere (14.6% CI: 10.3-18.8%) than Bamenda (6.0% CI: 4.5-7.5%). Furthermore more Fulani cattle (14.6%, CI: 10.3-18.8%) were IFN-γ positive than mixed breed (6.0% CI: 4.1-7.9%) cattle in Ngaoundere.

The proportion of TB lesion positive cattle was higher in Ngaoundere (11.3% CI: 9.3-13.4%) than Bamenda (4.0% CI: 2.8-5.1%) although there was no difference between proportions of IFN-γ positive cattle in the same abattoir. More Fulani cattle slaughtered in Ngaoundere (19.1% CI: 14.6-23.6%) were positive for TB lesions than in Bamenda (4.0% CI: 2.7-5.2%) and than mixed breed cattle in Ngaoundere (7.9% CI: 5.9-10.0%). These proportions were similar to IFN-γ positive proportions in the respective abattoirs.

The proportion of cattle *Fasciola* pathology positive in Bamenda (0.9% CI: 0.3-1.5%) was much lower than Ngaoundere (49.9% CI: 46.5-53.4%). This was the case overall and when the sampled population was subsetted. The proportion of Fulani cattle (61.0% CI: 54.6-67.4%) positive for *Fasciola* pathology was higher than for mixed breed (45.9% CI: 41.9-49.9%) cattle in Ngaoundere. It is worth noting that the proportion of cattle positive for IFN-γ, TB lesion and *Fasciola* pathology when analysed by individual DS (0-5), did not demonstrate any differences in trend compared to when re-catagorised (Results not displayed).

	Bamenda		Ngaoundere	
	IFN-gamma	n	IFN-gamma	n
OVERALL	6.0% (4.6-7.4%)	1105	8.6% (6.7-10.4%)	874
Subsets				
< 3 years	4.2% (2.1-6.4%)	1102	10.7% (5.2-16.1%)	865
>= 3 years	6.8% (5.0-8.6%)		8.2% (6.2-10.2%)	
Female	6.2% (4.0-8.5%)	1105	8.5% (6.5-10.4%)	873
Male	5.8% (4.0-7.6%)		10.0% (2.9-17.0%)	
Mixed breed	5.4% (2.0-8.9%)	1105	6.0% (4.1-7.9%)	869
Fulani	6.0% (4.5-7.5%)		14.6% (10.3-18.8%)	
Exotic	2.0% (0.0-55.1%)		[redacted]	
	Bamenda		Ngaoundere	
	TB lesion	n	TB lesion	n
OVERALL	4.0% (2.8-5.1%)	1129	11.3% (9.3-13.4%)	935
Subsets				
< 3 years	2.8% (1.1-4.5%)	1125	7.7% (3.1-12.3%)	923
>= 3 years	4.6% (3.1-6.1%)		12.0% (9.7-14.2%)	
Female	5.2% (3.1-7.3%)	1129	11.4% (9.2-13.5%)	932
Male	3.2% (1.9-4.5%)		11.4% (4.4-18.4%)	
Mixed breed	3.5% (0.8-6.3%)	1129	7.9% (5.9-10.0%)	930
Fulani	4.0% (2.7-5.2%)		19.1% (14.6-23.6%)	
Exotic	2.0% (0.0-52.2%)		[redacted]	
	Bamenda		Ngaoundere	
	Fasciola pathology	n	*Fasciola* pathology	n
OVERALL	0.9% (0.3-1.5%)	1115	49.9% (46.5-53.4%)	821
Subsets				
< 3 years	0.6% (0.0-1.3%)	1111	47.5% (38.4-56.5%)	811
>= 3 years	0.9% (0.1-1.6%)		50.4% (46.6-54.1%)	
Female	1.2% (0.1-2.2%)	1115	49.6% (46.0-53.2%)	819
Male	0.7% (0.1-1.4%)		52.1% (40.5-63.7%)	
Mixed breed	1.8% (0.0-3.8%)	1115	45.9% (41.9-49.9%)	816
Fulani	0.7% (0.2-1.3%)		61.0% (54.6-67.4%)	
Exotic	0.0% (0.0-52.2%)		[redacted]	

Table 4.5: **Prevalence of bovine tuberculosis, by IFN-gamma assay (≥0.1) and TB lesion identification, and *Fasciola* pathology in slaughtered cattle from Bamenda (Sampling between February-July 2012) and Ngaoundere abattoirs (Sampling between July-August 2013).**

4.3.5 Diagnostic disagreement: factors associated with IFN-gamma assay false positives and negatives

The potential reasons for disagreement, between the IFN-γ assay and TB lesion, identification were investigated. Although the level of agreement is similar between the IFN-γ assay and TB lesion identification in Bamenda and Ngaoundere, the potential risk factors for disagreement are investigated separately because the samples are markedly different by age,sex, breed and *Fasciola* pathology (section 4.3.1 and section 4.3.4). In particular, *Fasciola* pathology was highly correlated with abattoir.

Multi-variate logistic regression models were constructed to investigate the potential combination of factors associated with diagnostic disagreement between the IFN-γ assay ($\geq$0.1) and lesion identification at meat inspection; thus identifying factors influencing false positive and negative IFN-γ results. A positive cut-off of $\geq$0.1 was used for the IFN-γ assay as this had best agreement with TB lesion identification (Tables 4.3 and 4.4). BCS was not included as an explanatory variable in the models as it is more likely to be a result of having bTB. Also exotic cattle were removed from the disagreement analysis (Bamenda n=5) as they were few in number (Section 4.3.1) and inclusion lead to poor model fit. Potential interactions between sex and age (Figure 4.4) along with breed and presence of *Fasciola* pathology (Ngaoundere only; Table 4.5) were investigated during model selection.

IFN-gamma assay false positives

The data were subsetted to investigate diagnostic disagreement for being IFN-γ assay positive and TB lesion negative ("IFN-γ assay false positives") using two methods to produce two final models per abattoir:

- **Model a):** Subsetted by IFN-γ assay ($\geq$0.1) positive cattle and using TB lesion status as the dependent variable (Figure 4.1 a. and table 4.6 a.).
- **Model b):** Subsetted by TB lesion negative cattle and using IFN-γ assay status as the dependent variable (Figure 4.1 b. and table 4.6 b.).

The final selected models constructed for Bamenda and Ngaoundere abattoirs, did not identify any statistically significant (p value$\leq$0.05) risk factors to explain IFN-γ positive and TB lesion negative disagreement (Table4.6) (Appendix I).

BAMENDA			
(a) IFN-gamma assay POSITIVE subgroup (>=0.1, n=60)			
Model	**K**	**AIC**	**Δ AIC**
lesionANPN∼1 + sex + dentition + breed	4	76.93	0.00
lesionANPN∼1 + sex * dentition + breed	5	78.57	1.63
lesionANPN∼1	1	76.99	0.05
(b) TB Lesion NEGATIVE subgroup (n=1043)			
Model	**K**	**AIC**	**Δ AIC**
bovigam.01∼1 + sex + dentition + breed	4	351.03	3.23
bovigam.01∼1 + sex * dentition + breed	5	352.75	5.01
bovigam.01∼1	1	347.75	0.00
NGAOUNDERE			
(a) IFN-gamma assay POSITIVE subgroup (>=0.1, n=48)			
Model	**K**	**AIC**	**Δ AIC**
lesionANPN∼1 + sex + dentition + FgPathBin + breed	5	63.94	2.57
lesionANPN∼1 + sex * dentition + FgPathBin * breed	7	61.37	0.00
lesionANPN∼1 + sex + dentition + FgPathBin * breed	6	65.08	3.71
lesionANPN∼1 + sex * dentition + FgPathBin + breed	6	61.62	0.25
lesionANPN∼1	1	68.63	7.26
(b) TB Lesion NEGATIVE subgroup (n=687)			
Model	**K**	**AIC**	**Δ AIC**
bovigam.01∼1 + sex + dentition + FgPathBin + breed	5	214.41	1.09
bovigam.01∼1 + sex * dentition + FgPathBin * breed	7	215.36	2.03
bovigam.01∼1 + sex + dentition + FgPathBin * breed	6	216.44	3.11
bovigam.01∼1 + sex * dentition + FgPathBin + breed	6	213.33	0.00
bovigam.01∼1	1	216.76	3.43

Table 4.6: **Disagreement model selection to investigate risk factors for IFN-gamma assay false positives.**

Each of abattoir has two models to investigate IFN-gamma false positives: (a) Dependent variable TB lesion negative (lesionANPN) in IFN-γ positive sub group. (b) Dependent variable IFN-γ assay positive (bovigam.01) in TB lesion negative sub group. Explanatory variables included are sex (Sex), breed (Breed), dentition (Age), FgPathBin (Identification of *Fasciola* pathology at PME), OLS (Overall lesion score) and cultureR (TB lesion culture result). Key: Significant selected model= Grey; K= Number of parameters; AIC= Akaike information criterion; Δ AIC= Delta akaike information criterion; *= Interaction between variables.

IFN-gamma assay false negatives

Secondly results from slaughtered cattle were subsetted to investigate diagnostic disagreement for being IFN-γ assay negative and TB lesion positive ("IFN-γ assay false negatives") using two methods to produce two final models per abattoir:

Results from sampled cattle were also subsetted, for Bamenda and Ngaoundere, to investigate diagnostic test disagreement by being IFN-γ assay negative (<0.1) and TB lesion positive to produce two final models per abattoir:

- **Model c):** Subsetted by IFN-γ assay (<0.1) negative cattle and using TB lesion status as the dependent variable (Figure 4.2 c., tables 4.7 c. and 4.8 c.).
- **Model d):** Subsetted by TB lesion positive cattle and using IFN-γ assay status as the dependent variable (Figure 4.2 d. and table 4.7 d.).

Cattle sampled in Ngaoundere abattoir that are IFN-γ negative have increased odds of being TB lesion positive if they have evidence of *Fasciola* pathology and if they are Fulani breed (Ngaoundere model type c): Table4.8). Cattle that were both Fulani breed and had evidence of *Fasciola* pathology had increased the odds of being TB lesion positive but the negative interaction resulted in a smaller effect than expected from the individual effects. No risk factors for IFN-γ false negatives were identified for cattle slaughtered in Bamenda as final models do not have statistically significant explanatory variables (p value$\geq$0.05) (Appendix I).

BAMENDA			
(c) IFN-gamma assay NEGATIVE subgroup (>=0.1, n=1023)			
Model	**K**	**AIC**	**Δ AIC**
lesionANPN∼1 + sex + dentition + breed	4	229.09	0.00
lesionANPN∼1 + sex * dentition + breed	5	230.97	1.89
lesionANPN∼1	1	229.55	0.47
(d) TB Lesion POSITIVE subgroup (n=43)			
Model	**K**	**AIC**	**Δ AIC**
bovigam.01∼1 + sex + dentition + breed + OLS + cultureR	7	93.28	0.00
bovigam.01∼1 + sex * dentition + breed + OLS + cultureR	8	95.04	1.76
bovigam.01∼1	1	97.60	4.32
NGAOUNDERE			
(c) IFN-gamma assay NEGATIVE subgroup (>=0.1, n=714)			
Model	**K**	**AIC**	**Δ AIC**
lesionANPN∼1 + sex + dentition + FgPathBin + breed	5	410.14	3.41
lesionANPN∼1 + sex * dentition + FgPathBin * breed	7	407.03	0.31
lesionANPN∼1 + sex + dentition + FgPathBin * breed	6	406.72	0.00
lesionANPN∼1 + sex * dentition + FgPathBin + breed	6	410.33	3.60
lesionANPN∼1	1	409.20	2.48
(d) TB Lesion POSITIVE subgroup (n= 77)			
Model	**K**	**AIC**	**Δ AIC**
bovigam.01∼sex + dentition + FgPathBin + breed + OLS + cultureR	8	95.52	0.00
bovigam.01∼sex * dentition + FgPathBin * breed + OLS + cultureR	10	99.23	3.72
bovigam.01∼sex * dentition + FgPathBin + breed + OLS + cultureR	9	97.67	2.16
bovigam.01∼sex + dentition + FgPathBin * breed + OLS + cultureR	9	97.21	1.70
bovigam.01∼1	1	97.60	2.09

Table 4.7: **Disagreement model selection to investigate risk factors for IFN-gamma assay false negatives.**
(c) Dependent variable lesion positive (lesionANPN) in IFN-γ negative sub group. (d) Dependent variable IFN-γ assay negative (bovigam.01) in lesion positive sub group. Explanatory variables included are sex (Sex), breed (Breed), dentition (Age), FgPathBin (Identification of *Fasciola* pathology at PME), OLS (Overall lesion score) and cultureR (TB lesion culture result). Key: Significant selected model= Grey; K= Number of parameters; AIC= Akaike information criterion; Δ AIC= Delta akaike information criterion; *= Interaction between variables.

NGAOUNDERE						
(c) IFN-gamma assay NEGATIVE subgroup (>=0.1, n=714)						
TB Lesion		lesionANPN∼sex + dentition + FgPathBin * breed				
		Variables	Levels	Odds ratio	95% CI	p value
+	-	sex	Male	1		
			Female	1.01	0.42-3.03	0.98
		dentition	<3 years	1		
			>=3 years	1.66	0.74-4.43	0.26
59	655	breed	Mixed breed	1		
			Fulani	3.90	1.59-9.35	0.01
		FgPathBin	Negative	1		
			Positive	2.38	1.20-4.93	0.02
		breed*FgPathBin	Fulani*Positive	0.25	0.08-0.8	0.03

Table 4.8: **Final disagreement risk factor model for IFN-gamma assay false negatives.** (c) Dependent variable lesion positive (lesionANPN) in IFN-γ negative sub group for Ngaoundere abattoir (n=714). Key: lesionANPN= TB lesion result (Positive or negative); sex= Sex of cattle (Male or Female); dentition= Age of cattle by DS (<3 years or $\geq$3 years); FgPathBin= *Fasciola* pathology score; breed= Breed of cattle (Mixed breed or Fulani breed); *= Interaction between variables.

4.4 Discussion

This is the first time IFN-γ assay has been used to diagnose bTB in Cameroon and compared to TB lesion identification and *M. bovis* serology. The composition of the samples taken from two abattoirs are different with more younger (<3 years) male Fulani cattle in Bamenda and older ($\geq$3 years) female mixed breed cattle in Ngaoundere. Differences in TB lesion presentation were noted between Bamenda and Ngaoundere abattoirs highlighting the potential range of disease states in cattle. As these factors can influence development of immune responses to *M. bovis* (164; 374; 117) the two abattoirs are analysed separately.

Immunological and pathological progression of bTB is unlikely to be uniform between cattle in naturally infected populations (17; 164). Specifically, IFN-γ responses vary between individuals (161; 375), as bovine immune responses to *M. bovis* are affected by a variety of host, pathogen and diagnostic test factors that are specific to the cattle population tested (193). A low level of agreement (κ= 0.21-0.40 ("fair" agreement)) was observed between the IFN-γ assay and TB lesion identification at meat inspection ($\geq$0.1 positive cut-off value) in both Bamenda (κ= 0.33) and Ngaoundere (κ= 0.33). The "fair" agreement highlighted, between the IFN-γ assay and TB lesion identification, is likely to have been influenced by the stage of pathogenesis as it is well recognised that the IFN-γ responses to *M. bovis* change over the course of infection (Figure 1.2.6). Presence of *M. bovis* directly stimulates the CMI response to produce IFN-γ (167; 17) and the development of tuberculous lesions from as early as a week post-infection (161). In the early stages of infection, the IFN-γ response within TB lesions is similar to the peripheral IFN-γ responses detectable by in-vitro whole blood stimulation using the IFN-γ assay (159; 167). However as pathology progresses, *M. bovis* is often concentrated within

TB lesions of infected cattle (140). Production of IFN-γ is dependent on T-lymphocytes being stimulated by *M. bovis* antigens exposed to the host's immune system. As *M. bovis* antigen presentation is variable from within a TB lesion and into the systemic immune system (85; 255). IFN-γ and other CMI responses are likely to fluctuate depending upon the stage of pathogenesis and length of exposure to *M. bovis* (17). Also peripheral IFN-γ wane in latent and chronic *M. bovis* infections (130; 184), possibly due to their encapsulation within TB lesions. Hence despite IFN-γ responses and TB lesion development being part of the CMI response to *M. bovis*, and subsequent bTB disease, the two responses may not be directly correlated.

The "poor" to "fair" agreement of the IFN-γ assay ($\geq$0.1 positive cut-off value) and *M. bovis* serology (κ= 0.01-0.2), in Bamenda (κ= 0.03 CI: 0.01-0.06) and Ngaoundere (κ= 0.22 CI: 0.12-0.31), is possibly because the two tests are measuring different aspects of the immune response to *M. bovis*. Initially *M. bovis* infection stimulates a CMI response, including IFN-γ responses, with humoral immune responses developing in chronically infected cattle (164). In later stages of bTB cattle can become anergic, not producing CMI responses at all, despite having extensive TB lesion pathology (256; 258). Humeral responses can occur at the same time as IFN-γ responses, from 6-8 weeks post infection, and therefore serological responses may not always be restricted to chronic *M. bovis* infections or bTB (258). Thus the variation in *M. bovis* serology responses may partly explain the generalised poor agreement not only with IFN-γ responses but also the "poor" agreement with TB lesion identification in Bamenda (κ= 0.03 CI: 0.01-0.05) and Ngaoundere (κ= 0.2 CI: 0.11-0.29).

Although *M. bovis* serology agreement with the IFN-γ assay and TB lesion identification was "poor" in both abattoirs it was lower in Bamenda than Ngaoundere. As previously discussed this could be related to differences in chronicity of infections between the abattoirs with the proportion of older ($\geq$3 years) and female cattle was

higher in Ngaoundere than Bamenda. Although OLS was similar between the two abattoirs, it is ultimately unknown how long cattle have been infected with *M. bovis*. NTM infected cattle were present in both abattoirs and could have contributed to poor agreement with the IFN-γ assay and TB lesion identification (168; 376). The *M. bovis* antibody ELISA uses *M. bovis* MPB70 and MPB83 antigens to diagnose bTB (180; 377). Cross-reactions have been investigated with this ELISA in cattle infected with *M. avium paratuberculosis* (378) but other species of NTM have not been investigated. Other species of NTM were diagnosed, as part of the wider study (379), and this relationship could be investigated further in the future. Another potential reason for difference in agreement between the two abattoirs is that the *M. bovis* antibody ELISA testing was performed at separate times for Bamenda (October 2012) and Ngaoundere (March 2014). An unknown problem could have occurred with the laboratory protocol or ELISA kit, resulting in spurious results for either abattoir. Overall serological assays could be useful in combination with the IFN-γ assay for detecting anergic bTB positive cattle, but performance of the *M. bovis* antibody ELISA would require repeated trials in this setting.

IFN-γ or serological responses were not correlated with lesion severity in the abattoir study (quantified by OLS in this study). In an experimental study, 32x 6-month old calves inoculated with *M. bovis* intranasally and sequentially 4x slaughtered at 15, 28, 42, 60, 90, 180, 270, and 370 days after inoculation (70). TB lesions were inspected histologically and grossly to measure size of TB lesions. TB lesions isolated, from retropharyngeal LNs, did increase in size until day 28 and this plateaued. All calves developed large (4/4 calves; 1x TB lesion $\geq$10mm in diameter) and multicentric (4/4 calves; $\geq$1 TB lesion site) TB lesions in their retropharyngeal LNs between 80-370 day samplings. However in the abattoir study, cattle had evidence of multiple lesions in a high proportion of animals. Suggesting many cattle may have been infected for

long periods and a proportion are likely to have become anergic to *M. bovis* infection. Furthermore throughout *M. bovis* infection, lesions are likely to vary in size and maybe too small to detect on gross inspection in early stages of infection (85; 71) and some infected cattle may not develop lesions at all (263; 138), leading to lesion false negative results.

In human populations where TB is endemic, associations between prevalence of TB and age have been noted (380; 381). Age of cattle (measured by DS) could be a proxy for chronicity of *M. bovis* infection, yet the proportion of older cattle ($\geq$3 years) positive for TB lesions or IFN-γ responses was no different to younger cattle in either abattoir. Although it is generally accepted that prevalence of bTB increases with age in cattle in endemic settings (86; 87) as previously mentioned, latent infections may not always imply detectable IFN-γ responses or visible TB lesions (140; 3). Latent *M. bovis* infections are where *M. bovis* lies dormant and is not actively causing an immune response or disease. Latent infection of *M. bovis* or *M. tuberculosis* in people can result in active disease in older age groups due to a depression of immune responses leading to re-activation of latent infections (382). Latency in cattle is minimally studied cattle are generally slaughtered at a relatively young age and even in Cameroon there are few cattle $\geq$4 years of age. Hence although in other settings, including in humans, the prevalence of TB increases with age, the very skewed age structure of the abattoir sample and relative coarse age catagories by dentition scoring may be masking an underlying trend between TB prevalence and age. Longitudinal studies of *M. bovis* immune responses in naturally infected cattle would be especially useful. In respect to diagnostic performance, in settings where a spectrum of pathologies are present and to assist in the defining individual cattle as *M. bovis* or bTB disease positive.

It is worth mentioning that burden of *M. bovis* was unknown and length of infection

immeasurable in the abattoir study, with both being likely to vary between individual cattle naturally infected (17). Most of what is known about immune responses at various stages of *M. bovis* infection, comes from laboratory based experimental studies. Such studies infect cattle only study infection for a 1-12 months post infection in cattle between 6-18 months of age (258; 260; 70), in comparison to the wide age range of cattle studied in the abattoir study. Particularly with the majority of cattle in Ngaoundere being older ($\geq$3 years) females that may have been infected much longer. Laboratory-based studies also use larger infectious doses of *M. bovis* than would be expected with natural infections. For example in most laboratory studies, cattle are given *M. bovis* doses of $1x10^3$-5 $x10^5$ cfu (69; 70; 71) compared to 92cfu or less in natural infections (66). Larger doses of *M. bovis* in experimental studies can result in calves developing nasal turbinate lesions (69; 70; 71), a presentation not reported with natural infections. Hence the immunological profile and pathology described in laboratory-based studies, is potentially also not comparable to the likely variation in *M. bovis* burden and length of infection seen in Cameroonian cattle.

TB lesion identification at PME is reported to have a high specificity (48). Unlike peripheral immunological responses, visible gross TB lesions are unlikely to resolve once they have developed (135; 161) and thus presence of TB lesions imply that the animal has at least been infected at some point in its lifetime. In this study the majority of TB lesions cultured *M. bovis* with few of non-lesioned animals culturing *M. bovis* (379). This suggests a high specificity of TB lesion for *M. bovis* infection (138; 263). The apparent sensitivity of the IFN-γ assay was in both Bamenda (45.5% CI: 30.4-61.2%) and Ngaoundere (34.65% CI: 25.5-44.7%) much lower than reported in previous field studies (73-100% (Median: 87.6%)) (78). Subsequently comparing the IFN-γ assay to TB lesion identification to estimate diagnostic performance of the

IFN-γ assay was deemed suitable to investigate factors that may be associated with false negative results.

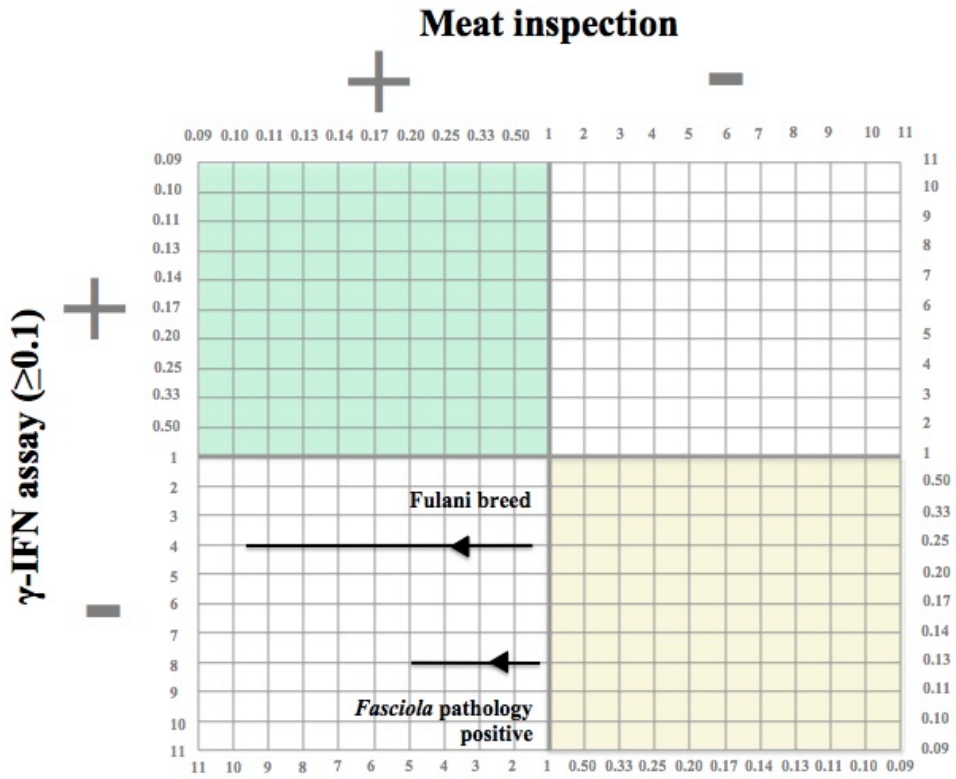


Figure 4.11: **Diagrammatic representation of summary of identified factors for IFN-gamma assay ($\geq$0.1) and lesion identification at meat inspection disagreement in Ngaoundere abattoir.**
Arrow direction and location is attributed to calculated OR with 95% CI (Table 4.8). The centre of the head of the arrow indicates the point estimate of the OR and the tips of the line indicate the limits of the 95% CI.

The presence of *Fasciola* pathology increases the odds of IFN-γ assay false negative results in cattle at Ngaoundere abattoir (Figure 4.11). It is possible that the effect associated with *Fasciola* pathology was confounded by another factor specific to Ngaoundere abattoir that was not measured during sampling, but obvious confounders such as age, sex and breed were investigated. *Fasciola hepatica* has been shown to suppress the IFN-γ response in BCG-immunised cattle (298) and although the mechanism is not completely eluded it has been demonstrated that *F. hepatica*

modulates the immune response from a Th1 (CMI) to a Th2 (Humeral) response (297; 383). Also liver TB lesions were only found in Ngaoundere and could imply a relationship between *Fasciola* and TB lesions at the site of *Fasciola* infection. Therefore presence of *Fasciola* infection may have implications for accurately describing bTB epidemiology with CMI diagnostics in naturally-infected populations. Although Ngaoundere abattoir was highly correlated with *Fasciola* pathology it is likely because of differences in prevalence of *Fasciola* at the time of sampling within the two study sites. In the UK, negative association has been demonstrated between areas of high *F. hepatica* prevalence and low bTB prevalence using the SCITT at a herd level (299). It is currently unclear which *Fasciola* species is present in Cameroon but it is suspected to be *F. gigantica* (212; 198). Less is known about the consequences of *F. gigantica* co-infection although it is suspected to have similar immune modulation properties to *F. hepatica* (287). Hence using the IFN-γ assay in Cameroon may underestimate bTB prevalence in the presence of *Fasciola* species infection.

In Ngaoundere abattoir, Fulani cattle breed are at increased odds of being IFN-γ assay negative/ lesion positive (Figure 4.11), although a similar relationship was not noted in Bamenda where there are more Fulani cattle. It might be that these cattle are kept longer, potentially being at increased risk of *M. bovis* infection for longer, resulting in waning of their IFN-γ responses (164). Other husbandry practices may influence immune responses to Fulani breed having odds of being IFN-γ assay false negatives (314). Differences in cattle husbandry might also account for the interaction between *Fasciola* pathology and Fulani breed. The relationship between bTB diagnosis and *Fasciola* in Cameroonian cattle will be explored further in chapter 5.

The specificity of the IFN-γ assay (Bamenda=95.7% CI: 94.3-96.8%; Ngaoundere=94.8% CI: 93.0-96.3%) was similar to that reported in previous studies

(85-99.6% (Median: 96.6%) (78)) but no risk factors were identified for IFN-γ assay false positives (Figure 4.11). A potential reason for false positive results, could be that as TB lesion identification sensitivity was low in the presence of early stage *M. bovis* infections (138; 163; 48). Although TB lesions can be present from as early as 14 days post infection in experimental studies (137) TB lesions do not always develop in earlier stages of pathogenesis where cattle are exposed to lower doses of bacilli which is common in natural infections(374; 66; 65; 71). Furthermore as TB lesion detection in abattoirs varies significantly (136; 384; 385) and rudimentary inspections may increase the proportion of TB lesion false negative cattle, thus increasing misdiagnosis of IFN-γ assay false positive results.

Including culture of NTMs in one of the IFN-γ disagreement models did not have a significant impact on odds of IFN-γ assay false positives. However not all cattle or tissues were cultured, reducing the sample size and subsequent power of this analysis. Classically NTM co-infections with *M. bovis* result in non-specific reactions and potentially leading to false positives in the IFN-γ assay (161; 166). The IFN-γ assay includes a control avian PPD (*M. avium*) in the protocol, to detect exposure to NTMs, which subsequently increases specificity of the test (163; 78; 386) thus reducing the probability of false positive results. Non-tuberculous mycobacteria of various different species have been shown to cross react with the avian and bovine PPDs, used in the IFN-γ assay, differently in different environments (166; 4). The impact on false positive rates of different NTM infections in some settings has warranted a different control PPD being added to the IFN-γ assay to minimise false positives (185; 387). Although the work is not included in this thesis, the NTMs were genotyped and a range of species were identified mainly consisting of *M. phlei* and *M. fortuitum* (379) from lesioned and non-lesioned cattle (the latter from retropharyngeal LNs). As it is unclear if the NTMs present in Cameroon will always cross-react with control avium

PPD used in the IFN-γ assay, further analysis from this dataset would be useful to investigate this interaction.

In conclusion as abattoir-based sampling of slaughtered cattle is not truly representative of the cattle population in Cameroon (Appendix H). Consequently this abattoir study should not be used to accurately describe bTB epidemiology in Cameroon. However the IFN-γ assay could be useful to highlight the significance of bTB in Cameroon but it is likely to underestimate the true prevalence of bTB due to the suspected poor sensitivity of the assay. Accounting for risk factors that decrease the sensitivity of the IFN-γ assay is vital to accurately describe bTB epidemiology when using this assay. Therefore the impact of *Fasciola* species on the IFN-γ assay bTB diagnosis will be investigated further (Chapter 5) prior to describing bTB epidemiology in Cameroonian cattle rearing communities (Chapter 8).

Chapter 5

Influence of Fasciola co-infection on bovine tuberculosis pathology and the performance of the interferon-gamma assay.

5.1 Introduction

Pathogens stimulate immune responses which can be predictable and useful in their diagnosis (388). Different pathogens stimulate and suppress host immune responses such as Th1 and Th2 type responses. However in natural populations, individuals are infected with multiple pathogens, or co-infections, rather than mono-infections (291). In the presence of multiple co-infections, the immune response detected for an individual pathogen is variable, depending upon the combination of infections along with their different interactions with the host immune system and each other (290). Trematodes, such as *Schistosoma* and *Fasciola* species, are of interest as they can actively modulate the host immune response to enable their survival with or without trematode-related-disease (389). The typical response is that trematodes down-regulate Th1 responses that control host CMI responses against them (276). However CMI are vital against other infections, particularly bacteria (254). Hence presence of trematode co-infections may influence development of bacterial disease within a host and have likely consequences to diagnosis and disease control.

Like many other infectious diseases, historically *M. bovis* infection, that can lead to development of bTB, has been studied in isolation as a single pathogen. In animals trematode co-infection with *F. hepatica* has been studied in a variety of host species to demonstrate evasion and modulation of the host immune responses including cattle (98). Throughout the course of infection *F. hepatica* down-regulates Th1 responses, with subsequent predominance of Th2 responses, in order to survive and replicate (297; 383; 275; 284; 283). Yet Th1 responses are important in protection against *M. bovis* (131). A reported bystander effect of *F. hepatica* co-infections is suppression of protective Th1 immune responses to *M. bovis* (285). Although the co-infection relationship has yet to be fully elucidated, various studies have demonstrated that *F. hepatica* co-infection has been associated with reduced Th1 immune responses (285),

mycobacterial burden, TB lesion pathology (301) and estimated bTB prevalence (299). This is particularly important when using the IFN-γ assay to detect bTB positive cattle. IFN-γ is a cytokine which is produced as part of the Th1 immune response to *M. bovis* (161). *Fasciola hepatica* co-infections down-regulate IFN-γ cytokine responses as the parasite down-regulates Th1 responses in the favour of Th2 responses such as IgG1 (298). When using the IFN-γ assay to diagnose bTB, presence of *F. hepatica* co-infection has lead to reduction of IFN-γ responses sufficiently below the positive cut off value leading to false negative diagnosis (302). However the extent of bTB misdiagnosis, using the IFN-γ assay, in bTB endemic *Fasciola* co-infected cattle with a range of bTB disease-states remains unquantified. Furthermore although similar immune evasion and modulation strategies have been identified in bovine *F. gigantica* infections, to *F. hepatica*, the effect of co-infection on bTB immune responses has been minimally investigated in any cattle population (272).

In Cameroon, *F. gigantica* infection is assumed to be the predominant *Fasciola* species infecting cattle (214; 217; 215) however this is yet to be investigated. Presence of *F. gigantica* or *F. hepatica* infections could account for false negative IFN-γ assay results which might underestimate bTB prevalence (Chapter 4). Although little research has been conducted with *F. gigantica* co-infections specifically and it is unclear whether the parasite has comparative effects on the host immune immune system as *F. hepatica*. Therefore presence of the species of *Fasciola* co-infections with *M. bovis* may need be taken into account when describing bTB epidemiology in Cameroon using the IFN-γ assay.

This chapter aims to describe the identification of *Fasciola* species isolated in Cameroon and prevalence of pathology in abattoir sampling. Along with the association between presence of *Fasciola* pathology and bTB is explored, including TB lesion pathology and IFN-γ responses. Finally the impact of co-infection on

detecting the presence of a bTB positive animal is quantified.

5.2 Materials and methods

Two samples will be analysed to address the aims of this chapter:

1. A sample of 60 *Fasciola* species parasites is used to determine the predominant species of *Fasciola* parasite infecting cattle in Cameroon.
2. Subsets of the cattle sampled from the abattoir study (Figure 5.6), are used to investigate the association between presence of *Fasciola* pathology and bTB diagnostic tests.

5.2.1 Classification of *Fasciola* species by RAPD-derived sequence PCR

A convenience sample of 60 *Fasciola* species parasites was collected from cattle slaughtered in four abattoirs in Cameroon during 2012-2013 (Figure 2.5). Although some parasites were sampled from the 2 abattoirs included in the abattoir study, the sampling was conducted separately:

1. Bamenda central municipal abattoir, NWR in February-July 2012 (n=35; including 20 from which ESP antigen was collected).
2. Ngaoundere central municipal abattoir, (VD) Adamawa Region in August 2013 (n=15).
3. Garoua central municipal abattoir, North Region in October 2013 (n=5).
4. Maroua central municipal abattoir, Extreme North Region in November 2013 (n=5).

The basic physical appearance of whole parasites was compared to gross descriptions of *F. hepatica* and *F. gigantica* parasites (390; 391; 199). Then speciation of *Fasciola*

parasites was conducted using two RAPD-derived sequence PCRs for *F. gigantica* and *F. hepatica* (n=60). Results were compared to determine the species of parasite. The method is fully described in subsection 3.4.1 of this thesis. Information about the cattle which the parasite's infected is only available for parasites where ESP antigen was collected (Chapter 6). The remainder of this chapter only investigates a proportion of the cattle sampled in the abattoir study.

5.2.2 Abattoir study design and diagnostic tests

The study design and sampling methodology of the abattoir study are described in detail in section 3.2.4 of this thesis. Diagnostic test methodology and interpretation formulae are described in sections 3.3 for bTB and 3.4 for *Fasciola*. A combination of bTB and *Fasciola* diagnostic tests were performed in the abattoir study (n=2064) which will be included in the analysis of this chapter:

1. Bovine tuberculosis lesion identification at meat inspection.
2. IFN-γ assay (Bovigam®). The positive cut-off value of $\geq$0.1 was chosen for the IFN-γ assay (Chapter 4).
3. *Fasciola* pathology identification at meat inspection.

Subsets of the cattle sampled are analysed in this chapter to investigate the co-infection relationship, outlined in figure 5.1.

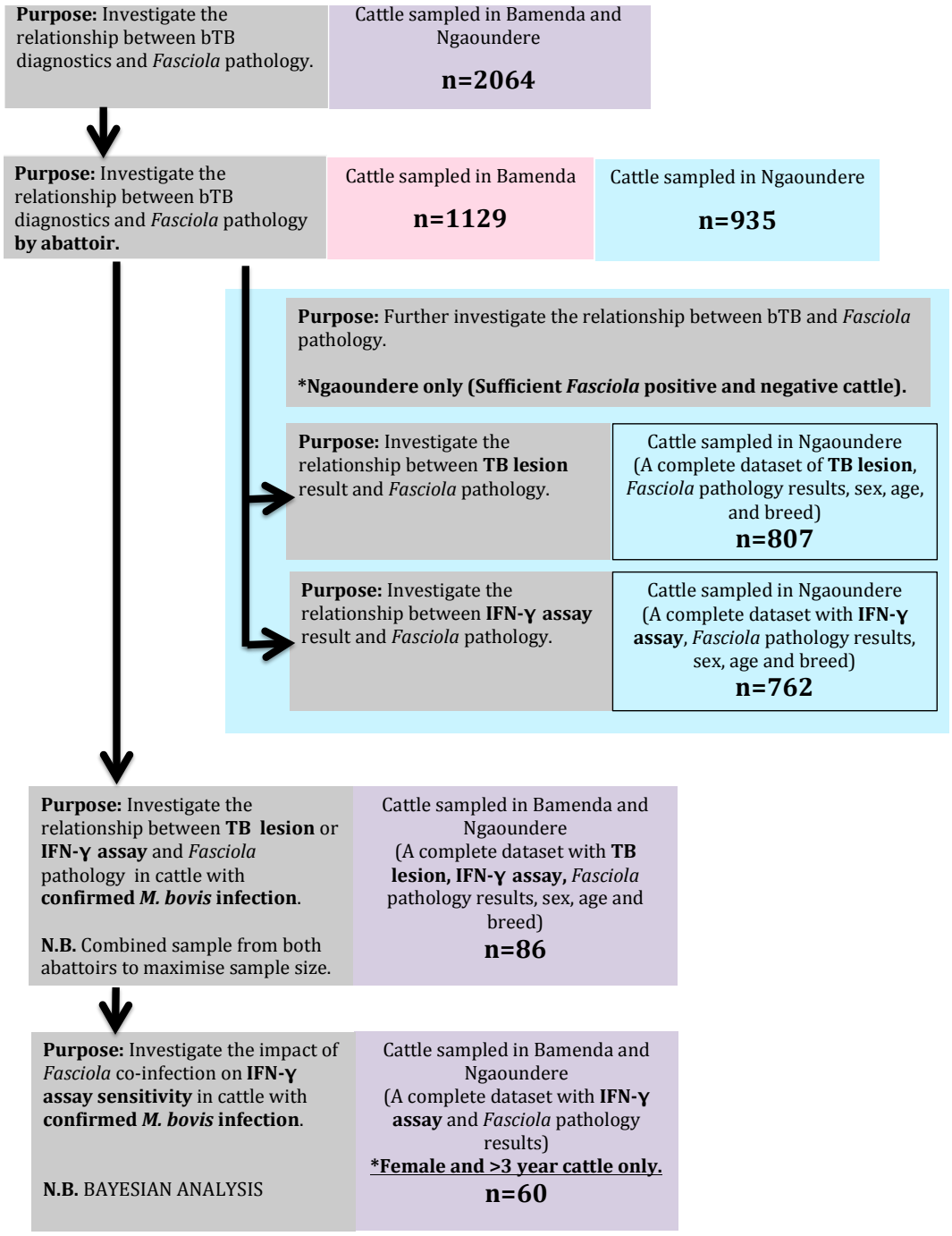


Figure 5.1: **A schematic diagram to explain the subsets used, from the abattoir sample, to investigate the co-infection relationship between bovine tuberculosis and *Fasciola* infection.**

Key: Sample drawn from; Both abattoirs= purple; Bamenda abattoir= pink; Ngaoundere abattoir= blue.

5.2.3 Statistical analysis

Fasciola species

The proportion of *Fasciola* species parasites sampled (n=60) is calculated with 95% CI calculated using exact binomial methods. Estimated proportions and CIs are calculated using the *svyby* of the *survey* package (346).

Descriptive statistics for co-infection for the abattoir study

Proportions of positive cattle for the IFN-γ assay, TB lesion identification, *Fasciola* pathology and co-infected are calculated for both abattoirs (n=2064), Bamenda (n=1129) and Ngaoundere (n=935) seperately. Cattle deemed co-infected are bTB positive by either TB lesion or IFN-γ assay with evidence of *Fasciola* pathology. Denominators vary depending upon whether complete datasets were available and all denominators are made clear in the text. Potential relationships between positive test results and individual animal variables such as DS, sex and breed is investigated in 2x2 tables. The DS variable is recategorised to "<3 years (DS<2)" and "$\geq$3 years (DS$\geq$2)". The breed variable is recategorised to mixed breed (Mixed breed and Gudali) and Fulani breed (Red and White Fulani) due to small numbers of cattle in some subsets (Red Fulani and Gudali breeds). Estimated proportions and CIs are calculated using the *svyby* of the *survey* package (346).

Relationship between *Fasciola* pathology with TB lesion and IFN-γ result

Subsets of data from the abattoir study using in co-infection analysis (Figure 5.1) and described here briefly. As very few cattle were *Fasciola* pathology positive in Bamenda, association with bTB test results is investigated using the Ngaoundere

dataset (n=935; Chapter 4 and figure 5.1). Due to missing data for different diagnostic test and animal level variable combinations data subsets from the Ngaoundere with recorded TB lesion (n=807), IFN-γ assay (n=762) and animal level variables (Sex, age and breed) are used in the analysis.

Multivariable logistic regression (MLR) models were used to investigate the co-infection relationship (Chapter 3). Firstly the relationship between being TB lesion positive and *Fasciola* pathology was investigated using MLR models (n=807). The outcome variable was the binary result of TB lesion positive (1) or negative (0). Secondly the relationship between being IFN-γ positive and *Fasciola* pathology was investigated using MLR models (n=762). The outcome variable was the binary result of IFN-γ positive (1) or negative (0). Animal level explanatory variables were always included in model selection to control for confounding. Explanatory variables include age (DS), sex and breed of cattle with potential interactions were taken into account. Principles of MLR model selection are outlined in chapter 3. All models included all explanatory variables, model selection included the different possible interactions between explanatory variables. Model selection was based on the AIC and the best model, to fit the dataset, was selected using the lowest AIC and Δ AIC to confirm model selection (360). MLR models were constructed using *glm* function in the *stats* package (343) with AIC and Δ AIC calculated using the *modavg* function from the *AICcmodavg* package (363).

A subset of *M. bovis* culture positive (n=86) cattle, from both Bamenda and Ngaoundere abattoirs, was also used to investigate the association between OLS (Overall lesion score (Chapter 4)), the raw continous IFN-γ response and *F. gigantica* pathology (Figure 5.1). A dataset combination of both abattoirs was used due to the limited number of *M. bovis* culture positive cattle available in the abattoir study (Bamenda: 30; Ngaoundere: 56) with a complete set of diagnostic test results and

animal level variables recorded. Primarily OLS score and the raw continous IFN-γ response was used to investigate the relationship between size of TB lesion and presence of *Fasciola* pathology. These relationships were provisionally explored using scatter plots then the statistical significance investigated students T test (348) calculated using the *stats* package (343).

Estimation of *Fasciola* co-infection effect on IFN-γ assay sensitivity

This analysis was conducted by Dr B M de C Bronsvoort and a brief explanation is included for completeness. A subset of the *M. bovis* culture positive cattle (n=86) was first used to evaluate the IFN-γ test sensitivity. The sub-group consisted of female cattle $\geq$3 years (n=60), as this was the largest subgroup and the purpose was to assess the sensitivity without the potential confounding and interactions from sex and age (DS) (Figure 5.6). The IFN-γ assay sensitivity and specificity was estimated relative to the *M. bovis* culture and typing positivity. The statistical uncertainty was estimated with a Bayesian approach assuming a uniform (0, 1) prior distribution for sensitivity and specificity, and a binomial likelihood. For example, the posterior distribution for sensitivity where r animals were IFN-γ positive out of a total of n animals that were *M. bovis* culture and type positive is beta(r +1, n - r + 1). The 2.5 and 97.5 percentile points of this distribution provide a 95% Bayesian credible interval for diagnostic sensitivity. The point estimate of sensitivity is r / n. The Bayesian binomial estimator was constructed using the *rjags* package (392) using 5000 iterations burnin and 10,000 adaptive iterations. The simplified model is give below:

$$r_1 \sim binom(Se_{F+ve}, n_1)$$

$$r_2 \sim binom(Se_{F-ve}, n_2) \quad (5.1)$$

where r_1 was 9 and n_1 was 27 and r_2 was 17 and n_2 was 33.

5.3 Results

5.3.1 *Fasciola* species identified

On gross appearance all parasites had characteristics of *F. gigantica* including slender shoulders and a long tapered shape (Figure 5.2) (390; 391; 199). Subsequently all *Fasciola* parasites tested by RAPD-derived sequence PCR were identified as *F. gigantica* species (100%, CI: 94.0-100%, n=60) (Figure 5.3) For the remainder of this chapter *Fasciola* pathology related results are subsequently referred to as *F. gigantica* pathology.

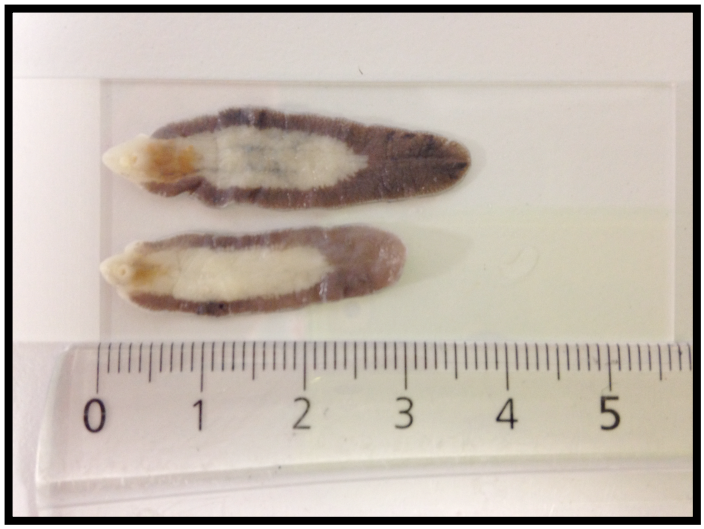


Figure 5.2: **An image of two of the *Fasciola* parasites sampled (n=60).** Measurement in cm.

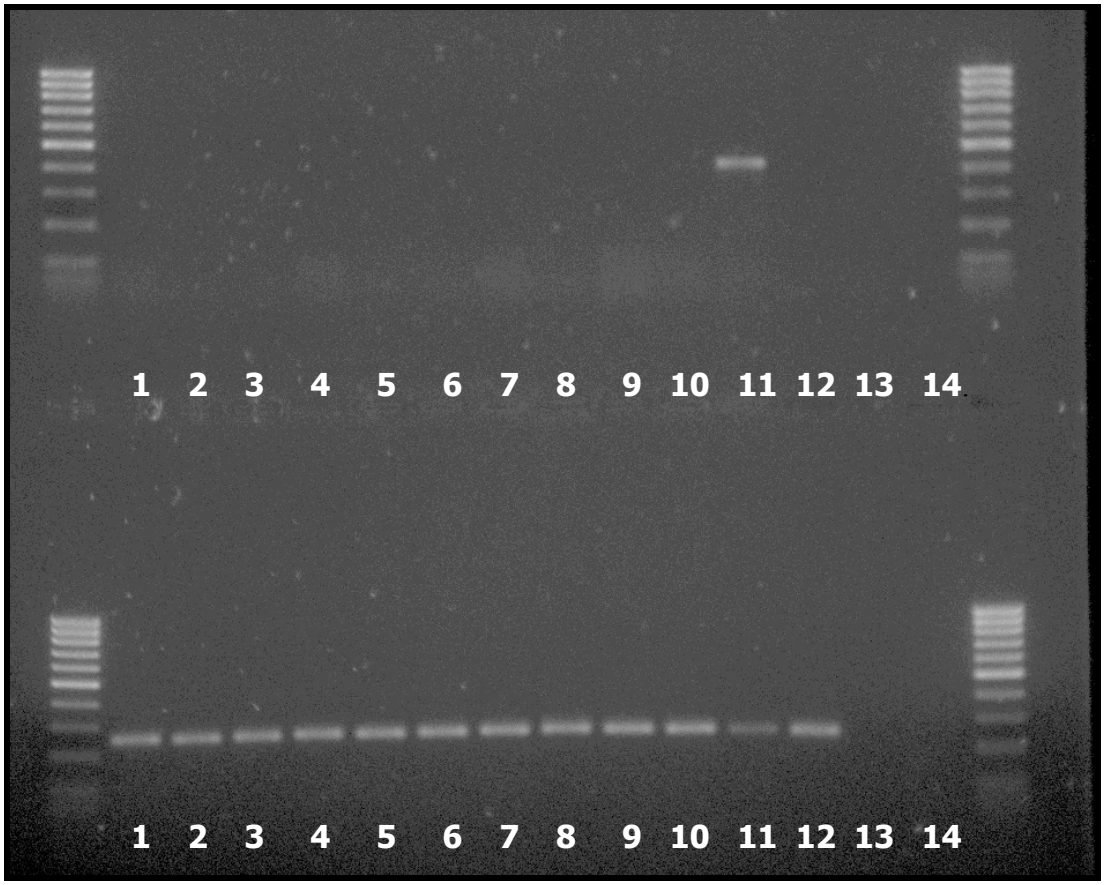


Figure 5.3: **Image of an example RAPD *Fasciola* species PCR gel UV trans-illuminator.** *Fasciola* DNA from 10 unknown *Fasciola* species samples are in columns 1- 10. Column 11 is the *F. hepatica* positive control. Column 12 is the *F. gigantica* positive control. Columns 13 and 14 are bovine DNA and molecular grade water as negative controls. DNA ladders (100 bp) are present left and right of columns 1 and 14 respectively. The top PCR uses a set of primers for only *F.hepatica*. The lower PCR uses a set of primers *F.gigantica*. Both primer sets have to be run for each tested sample to determine whole *Fasciola* parasite species by comparison. DNA samples which are *F.hepatica* show one band on each PCR (Column 11 *F. hepatica* positive control). DNA samples which are *F.gigantica* show only a band on the *F. gigantica* PCR: (Columns 1-10 unknown samples and Column 12 *F.gigantica* positive control).

5.3.2 Impact of *F. gigantica* on bTB pathology and diagnosis

Proportion of bTB and *F. gigantica* positive cattle

The proportion of cattle sampled in the abattoir study, subsetted by abattoir, is summarised in Table 5.1. Proportions of cattle sampled inspected for TB lesions, *F. gigantica* pathology and tested using the IFN-γ assay are described. All cattle sampled, in Bamenda and Ngaoundere abattoirs, were inspected for TB lesions. It was not always possible to collect samples or data from all cattle sampled due to logistics of sampling within the abattoirs. Hence not all cattle were blood sampled to conduct the IFN-γ assay and the result of meat inspection for *F. gigantica* pathology was not always recorded. In Bamenda more cattle were IFN-γ assay positive than TB lesion positive. In Ngaoundere more cattle were TB lesion positive than IFN-γ assay positive. Overall Ngaoundere had more cattle classed TB lesion and IFN-γ assay positive than Bamenda. Furthermore the proportion of cattle identified positive for *F. gigantica* pathology was markedly greater in Ngaoundere than Bamenda. In Ngaoundere 60.0% of TB lesion positive and 49.2% of IFN-γ positive cattle also had evidence of *F. gigantica* pathology. Due to few cattle being positive for *F. gigantica* pathology in Bamenda abattoir, only cattle from Ngaoundere abattoir were used to investigate the relationship between TB lesion, IFN-γ responses and *F. gigantica* pathology.

	Bamenda abattoir	**Ngaoundere abattoir**	**Total abattoir**
Number sampled	1129	935	2064
TB lesion inspected	100% (1129/1129)	100% (935/935)	100% (2064/2064)
IFN-gamma tested	97.87% (1105/1129)	93.48% (874/935)	95.88% (1979/2064)
F. gigantica inspected	98.76% (1115/1129)	87.81% (821/935)	93.80% (1936/2064)
TB lesion positive	3.99% (45/1129)	11.34% (106/935)	7.32% (151/2064)
IFN-gamma positive	5.97% (66/1105)	8.58% (75/874)	7.12% (141/1979)
F. gigantica positive	0.09% (10/1115)	49.94% (410/821)	21.69% (420/1936)
Co-infected TB lesion positive	0.00% (0/45)	60.00% (51/85)	39.23% (51/130)
Co-infected IFN-gamma positive	3.17% (2/65)	49.18% (30/61)	25.40% (32/126)

Table 5.1: **Summary of proportions of sampled bTB, *F. gigantica* positive and co-infected cattle by abattoir.**

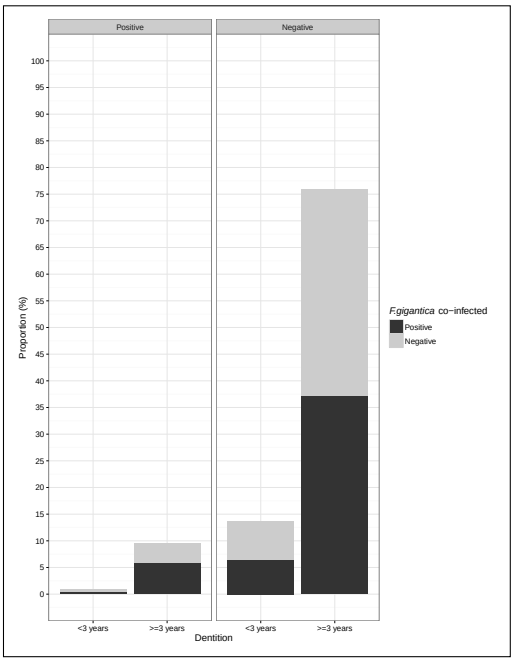
Association of *F. gigantica* with TB lesions

Initially, to investigate the association between TB lesions and *F. gigantica* pathology cattle were stratified by animal-related variables that could influence development of TB lesions and thus be potential confounders such as age (DS), sex and breed (Table 5.2 and figure 5.4). A subset of the Ngaoundere abattoir sample (n=807) was used for analysis. Consistently for age (DS), sex and breed over half of TB lesion positive cattle were also *F. gigantica* pathology positive with odds ratios displayed for completeness. To investigate this relationship further multivariable logistic regression (MLR) was used to estimate the association between the probability of having a TB lesion and *F. gigantica* pathology accounting for animal-related variables that could influence TB lesion development (n=807). Dentition score, sex, breed and *F. gigantica* pathology result were controlled for in all MLR models as fixed effects and their potential interactions. The best fit MLR model was selected using Δ AIC (Table 5.3) and the final model is given in Table 5.7. This model shows that there is a strong and statistically significant association with *F. gigantica* pathology, with co-infected animals having more than twice the odds of having a visible TB lesion. Also Fulani cattle were nearly 6 times more likely to be TB lesion positive than mixed breed cattle. The interaction between the Fulani breed and *F. gigantica* positive cattle was also statistically significant.

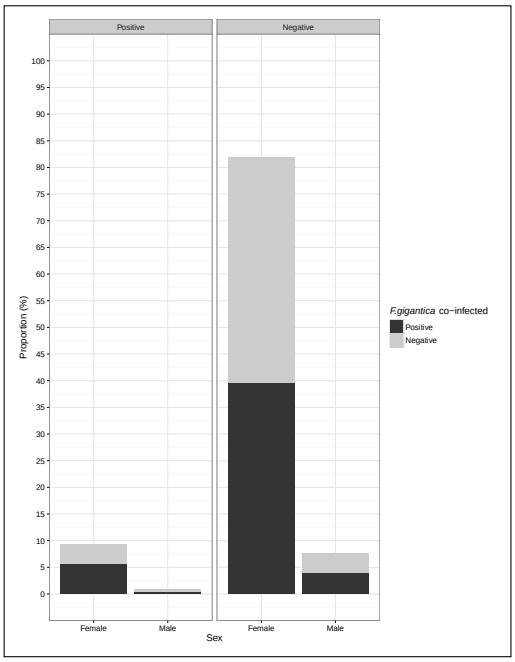
To assess the association between the "severity" of TB lesion observed and the presence of *F. gigantica* pathology. The subset of *M. bovis* culture positive cattle (n=86; Figure 5.1) were examined (Table 5.5). The 4 TB lesion scores were combined to give each animal an OLS, as described in Chapter 4, which was then compared to *F. gigantica* pathology status (n=86; Figure 5.5). No evidence of an association between being *F. gigantica* positive and OLS (p=0.77).

TB lesion Ngaoundere (n= 807)							
AGE (DS)							
< 3 years (n=118)				>= 3 years (n=689)			
TB Lesion							
	■	+	-		■	+	-
F. gigantica pathology	+	5	51	*F. gigantica* pathology	+	47	300
	-	4	58		-	30	312
OR=1.38 (CI: 0.39-4.90)				OR=1.54 (CI:1.00-2.38)			
SEX							
Male (n=71)				Female (n=736)			
TB Lesion							
	■	+	-		■	+	-
F. gigantica pathology	+	5	32	*F. gigantica* pathology	+	47	319
	-	4	30		-	30	340
OR=1.15 (CI: 0.34-3.43)				OR=1.54 (CI: 1.03-2.45)			
BREED							
Mixed breed (n=585)				Fulani (n=222)			
TB Lesion							
	■	+	-		■	+	-
F. gigantica pathology	+	31	237	*F. gigantica* pathology	+	21	114
	-	15	302		-	19	68
OR=2.44 (CI: 1.35-4.43)				OR=0.71 (CI: 0.41-1.25)			

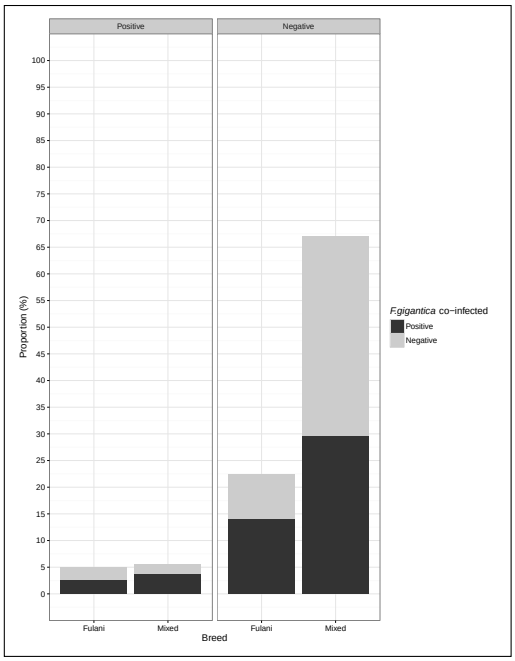
Table 5.2: **2x2 tables of cattle sampled in Ngaoundere abattoir by TB lesion and *F. gigantica* pathology result stratified by age (DS), sex and breed (n=807).**
Cattle sampled are only included with complete datasets for TB lesion result, *F. gigantica* result, age (DS), sex and breed. Key: OR= Odds ratio.



(a) Dentition score



(b) Sex



(c) Breed

Figure 5.4: **Proportions of cattle sampled, in Ngaoundere abattoir, TB lesion and *F. gigantica* pathology positive by dentition score, sex and breed (n=807).**

Ngaoundere TB lesion model selection (n=807)			
Model	**K**	**AIC**	**Δ AIC**
lesionANPN∼sex + dentition + FgPathBin + breed	5	538.14	6.87
lesionANPN∼sex * FgPathBin + breed + dentition	6	539.92	8.66
lesionANPN∼sex * breed + FgPathBin + dentition	6	539.93	8.66
lesionANPN∼sex * dentition + breed + FgPathBin	6	536.98	8.71
lesionANPN∼sex * FgPathBin + breed + dentition * sex	7	541.80	10.53
lesionANPN∼sex * FgPathBin + dentition + breed * sex	7	541.80	10.53
lesionANPN∼FgPathBin + sex * dentition + breed * sex	7	541.78	10.51
lesionANPN∼sex * FgPathBin + sex * dentition + breed * sex	8	543.68	12.41
lesionANPN∼FgPathBin * breed + sex + dentition	**6**	**531.27**	**0.00**
lesionANPN∼FgPathBin * dentition + sex + breed	6	540.17	8.90
lesionANPN∼FgPathBin * dentition + sex + breed * FgPathBin	7	533.30	2.03
lesionANPN∼FgPathBin * dentition + breed + sex * FgPathBin	7	541.96	10.69
lesionANPN∼FgPathBin * breed + dentition + sex * FgPathBin	7	533.27	2.00
lesionANPN∼sex * FgPathBin + FgPathBin * dentition + breed * FgPathBin	8	535.31	4.04
lesionANPN∼sex + FgPathBin + breed * dentition	6	539.69	8.43
lesionANPN∼sex * dentition + breed * dentition + FgPathBin	7	541.62	10.35
lesionANPN∼sex * dentition + FgPathBin * dentition + breed	7	542.01	10.74
lesionANPN∼breed * dentition + FgPathBin * dentition + sex	7	541.73	10.46
lesionANPN∼breed * dentition + FgPathBin * dentition + sex * dentition	8	543.66	12.39
lesionANPN∼breed * dentition + FgPathBin * breed + sex * breed	8	534.44	3.17
lesionANPN∼sex * FgPathBin * breed * dentition	16	548.29	17.03

Table 5.3: **Multivariable logistic model selection for TB lesion association with *F. gigantica* pathology.**
Based on AIC showing the full model with the interaction between dentition and fluke status (n=807). Key: Grey= Selected model; K= Number of parameters; AIC= Akaike information criterion; Δ AIC= Delta akaike information criterion. Key: lesionANPN= TB lesion result (Positive or negative); sex= Sex of cattle (Male or Female); dentition= Age of cattle by DS (<3 years or ≥3 years); FgPathBin= *F. gigantica* pathology score; breed= Breed of cattle (Mixed breed or Fulani breed); *= Interaction between variables.

Ngaoundere TB lesion selected model (n=807)				
Variable	**Levels**	**Odds ratio**	**95% CI**	**p value**
sex	Male	1		
	Female	0.90	0.44-2.04	0.79
dentition	<3 years	1		
	>=3 years	1.58	0.80-3.51	0.22
breed	Mixed breed	1		
	Fulani	5.71	2.76-12.00	<0.01
FgPathBin	Negative	1		
	Positive	2.65	1.42-5.16	<0.01
breed*FgPathBin	Fulani*Positive	0.24	0.09-0.61	<0.01

Table 5.4: **Final model for the presence of TB lesions at slaughter and their association with the *F. gigantica* pathology (n=807).**
Key: lesionANPN= TB lesion result (Positive or negative); sex= Sex of cattle (Male or Female); dentition= Age of cattle by DS (<3 years or $\geq$3 years); FgPathBin= *F. gigantica* pathology score; breed= Breed of cattle (Mixed breed or Fulani breed); *= Interaction between variables.

M. bovis culture positive subset (n=86)						
AGE (DS)						
< 3 years (n=12)			>= 3 years (n=74)			
IFN-gamma						
	■	+	-		■	+	-
F. gigantica pathology	+	1	1	*F. gigantica* pathology	+	9	20
	-	2	8		-	25	20
OR=2.5 (CI: 0.39-16.05)				OR=0.56 (CI: 0.3-1.02)			
SEX						
Male (n=18)				Female (n=68)			
IFN-gamma						
	■	+	-		■	+	-
F. gigantica pathology	+	1	1	*F. gigantica* pathology	+	10	19
	-	10	6		-	17	22
OR=0.8 (CI: 0.19-3.37)				OR=0.79 (CI: 0.43-1.46)			
BREED						
Mixed breed (n=31)				Fulani (n=55)			
IFN-gamma						
	■	+	-		■	+	-
F. gigantica pathology	+	3	12	*F. gigantica* pathology	+	7	9
	-	7	9		-	20	19
OR=0.46 (CI: 0.14-1.45)				OR=0.85 (CI: 0.45-1.61)			

Table 5.5: **2x2 tables of *M. bovis* culture positive cattle by IFN-gamma assay and *F. gigantica* pathology binary result stratified by age (DS), sex and breed (n=86).**
Cattle sampled are only included with complete datasets for TB lesion result, *F. gigantica* result, age (DS), sex and breed. Key: OR= Odds ratio.

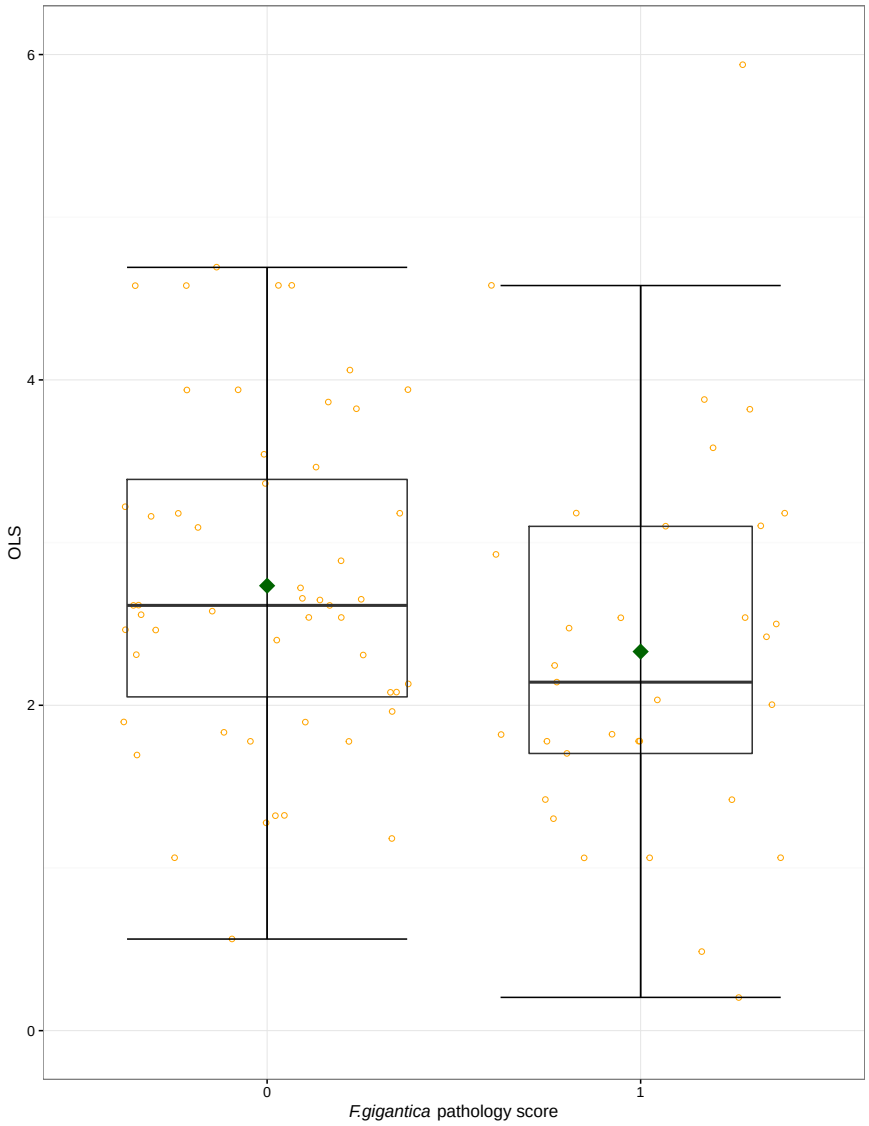


Figure 5.5: **Overall TB lesion (OLS) scores stratified by *F. gigantica* status**
In a subset of *M. bovis* culture positive cattle (n=86). Key: Mean= Green diamond; Individual cattle; Orange circles.

Impact of *F. gigantica* co-infection on IFN-γ response to bTB

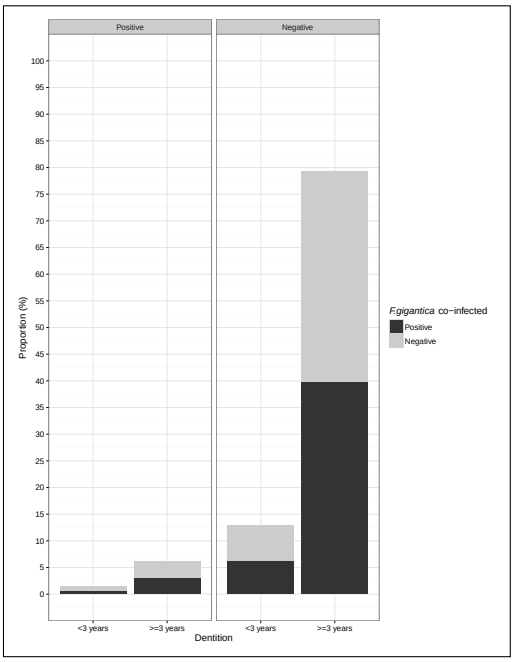
The association between IFN-γ result and *F. gigantica* pathology status was investigated. A subset of the Ngaoundere abattoir dataset was used (n=762) only including cattle with both diagnostic test results and animal data recorded. Cattle were stratified by animal-related variables that could influence development of IFN-γ responses (Table 5.6 and figure 5.6). For age (DS), sex and breed groupings, less than or equal to half of IFN-γ assay positive cattle were also *F. gigantica* positive. To investigate the relationship between IFN-γ and *F. gigantica* pathology result another MLR model was developed (n=762). Dentition score, sex, breed and *F. gigantica* pathology result were included in MLR models as fixed effects and their potential interactions included in model selection. Initially the best fit MLR model was selected using Δ AIC (Table 5.7 Δ AIC=0.0) however the best fit model displayed poor fit. The final model selected, given in Table 5.8, was selected with the lowest possible Δ AIC that satisfied model diagnostic criteria (Δ AIC=1.47 <2.0 indicates substantial support from the data (360)). Fulani cattle were 3 times more likely to be IFN-γ positive than mixed breed cattle ($p<0.05$). Although there was no statistically significant association between IFN-γ and *F. gigantica* pathology status ($p=0.14$).

It is possible the relationship observed with TB lesion, used as the proxy for infection with *M. bovis*, and *F. gigantica* pathology was being obscured by the immune modulation of *F. gigantica* and suppression of the IFN-γ response. Resulting in some cattle being defined as IFN-γ negative and masking any detectable association. Hence a subset of cattle with TB lesions confirmed as *M. bovis* positive by culture. A total of 86 cattle were positive for *M. bovis* at culture and the distribution of their IFN-γ assay results was plotted by *F. gigantica* pathology result (Figure 5.7). The plot suggests a depressed IFN-γ response in co-infected cattle compared to cattle only infected with *M. bovis*.

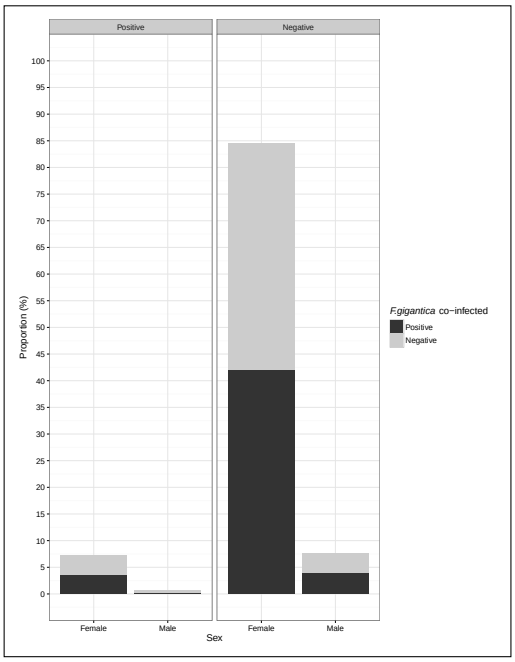
In order to estimate the impact of *F. gigantica* co-infection on IFN-γ responses, the binary diagnostic cut-off ($\geq$0.1) was applied to convert the result to a binary results and the new data compared to *F. gigantica* status in the subset of cattle with confirmed *M. bovis* culture positive. To avoid sample bias from the two abattoirs, with $\geq$3 year old females predominating in Ngaoundere and <3 year males in Bamenda (Chapter 4), a sub-group of cattle was selected for analysis (n=60). Only female cattle $\geq$3 years was used (n=60) as this was the largest subgroup and the purpose was to assess the sensitivity without the potential confounding and interactions from sex and age (DS). The proportion testing positive depending on *F. gigantica* status was approximated using a Bayesian estimator (Conducted by Dr B M de C Bronsvoort). The IFN-γ test sensitivity in *F. gigantica* negative populations was estimated to be 51.7% (95% highest density interval (HDI): 39.1-64.4%) and in *F. gigantica* positive populations this drops significantly by 20.3% (the 95% HDI of the difference was 0.1-39.4%) to 31.4% (95% HDI: 17.5-47.1%).

Ngaoundere IFN-gamma (n= 762)							
AGE (DS)							
< 3 years (n=111)				>= 3 years (n=651)			
IFN-gamma				IFN-gamma			
	[redacted]	+	-		[redacted]	+	-
F. gigantica pathology	+	1	52	*F. gigantica* pathology	+	20	307
	-	5	53		-	22	302
OR=0.21 (CI: 0.03-1.81)				OR=0.90 (CI: 0.50-1.62)			
SEX							
Male (n=64)				Female (n=698)			
IFN-gamma				IFN-gamma			
	[redacted]	+	-		[redacted]	+	-
F. gigantica pathology	+	1	31	*F. gigantica* pathology	+	20	328
	-	3	29		-	24	326
OR=0.33 (CI: 0.04-3.04)				OR=0.84 (CI: 0.47-1.49)			
BREED							
Mixed breed (n=553)				Fulani (n=209)			
IFN-gamma				IFN-gamma			
	[redacted]	+	-		[redacted]	+	-
F. gigantica pathology	+	9	240	*F. gigantica* pathology	+	12	119
	-	15	289		-	12	66
OR=0.73 (CI: 0.33-1.65)				OR=0.60 (CI: 0.28-1.26)			

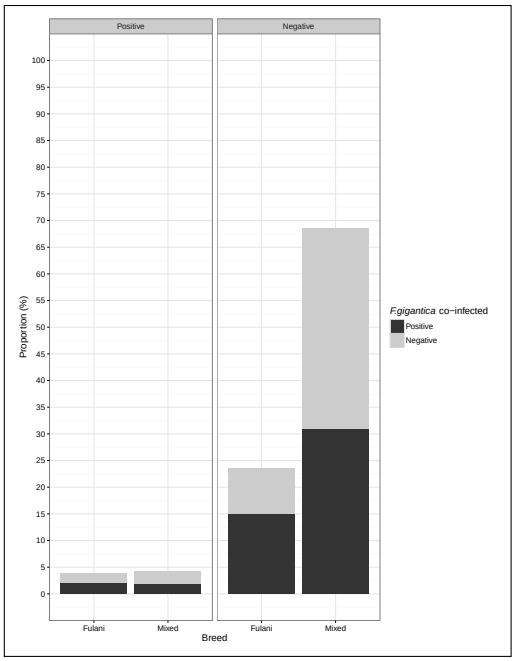
Table 5.6: **2x2 tables of cattle sampled in Ngaoundere abattoir by IFN-gamma assay and *F. gigantica* pathology binary result stratified by age (DS), sex and breed (n=762).** Cattle sampled are only included with complete datasets for TB lesion result, *F. gigantica* result, age (DS), sex and breed. Key: OR= Odds ratio.



(a) Dentition score



(b) Sex



(c) Breed

Figure 5.6: **Proportions of cattle sampled, in Ngaoundere abattoir, IFN-gamma and *F. gigantica* pathology positive by dentition score, sex and breed (n=762).**

Ngaoundere IFN-gamma model selection (n=762)			
Model	**K**	**AIC**	**Δ AIC**
bovigam.01∼sex + dentition + FgPathBin + breed	**5**	**354.09**	**1.47**
bovigam.01∼sex * FgPathBin + breed + dentition	6	355.27	2.65
bovigam.01∼sex * breed + FgPathBin + dentition	6	352.62	0.00
bovigam.01∼sex * dentition + breed + FgPathBin	6	355.96	3.34
bovigam.01∼sex * FgPathBin + breed + dentition * sex	7	357.08	4.46
bovigam.01∼sex * FgPathBin + dentition + breed * sex	7	352.80	0.18
bovigam.01∼FgPathBin + sex * dentition + breed * sex	7	354.52	1.90
bovigam.01∼sex * FgPathBin + sex * dentition + breed * sex	8	354.69	2.07
bovigam.01∼FgPathBin * breed + sex + dentition	6	355.93	3.31
bovigam.01∼FgPathBin * dentition + sex + breed	6	354.61	1.99
bovigam.01∼FgPathBin * dentition + sex + breed * FgPathBin	7	356.43	3.81
bovigam.01∼FgPathBin * dentition + breed + sex * FgPathBin	7	355.92	3.30
bovigam.01∼FgPathBin * breed + dentition + sex * FgPathBin	7	357.18	4.56
bovigam.01∼sex * FgPathBin + FgPathBin * dentition + breed * FgPathBin	8	357.81	5.19
bovigam.01∼sex + FgPathBin + breed * dentition	6	352.82	0.20
bovigam.01∼sex * dentition + breed * dentition + FgPathBin	7	354.33	1.71
bovigam.01∼sex * dentition + FgPathBin * dentition + breed	7	356.34	3.72
bovigam.01∼breed * dentition + FgPathBin * dentition + sex	7	353.56	0.94
bovigam.01∼breed * dentition + FgPathBin * dentition + sex * dentition	8	354.94	2.32
bovigam.01∼breed * dentition + FgPathBin * breed + sex * breed	8	352.87	0.25
bovigam.01∼sex * FgPathBin * breed * dentition	16	362.95	10.33

Table 5.7: **Multivariable logistic model selection for IFN-gamma positivity association with *F. gigantica* pathology.**
Based on AIC showing the full model with the interaction between dentition and fluke status (n=762). Key: Grey= Selected model; K= Number of parameters; AIC= Akaike information criterion; Δ AIC= Delta akaike information criterion. Key: bovigam.01= IFN-γ assay result (Positive or negative); sex= Sex of cattle (Male or Female); dentition= Age of cattle by DS (<3 years or $\geq$3 years); FgPathBin= *F. gigantica* pathology score; breed= Breed of cattle (Mixed breed or Fulani breed); *= Interaction between variables.

Ngaoundere IFN-gamma selected model (n=762)				
Variable	**Levels**	**Odds ratio**	**95% CI**	**p value**
sex	Male	1		
	Female	1.23	0.47-4.26	0.70
dentition	<3 years	1		
	>=3 years	1.19	0.53-3.22	0.70
breed	Mixed breed	1		
	Fulani	3.15	1.72-5.81	<0.01
FgPathBin	Negative	1		
	Positive	0.63	0.34-1.15	0.14

Table 5.8: **Final model for IFN-gamma positivity and association with the *F. gigantica* pathology (n=762).**

Key: bovigam.01= IFN-γ assay result (Positive or negative); sex= Sex of cattle (Male or Female); dentition= Age of cattle by DS (<3 years or $\geq$3 years); FgPathBin= *F. gigantica* pathology score; breed= Breed of cattle (Mixed breed or Fulani breed).

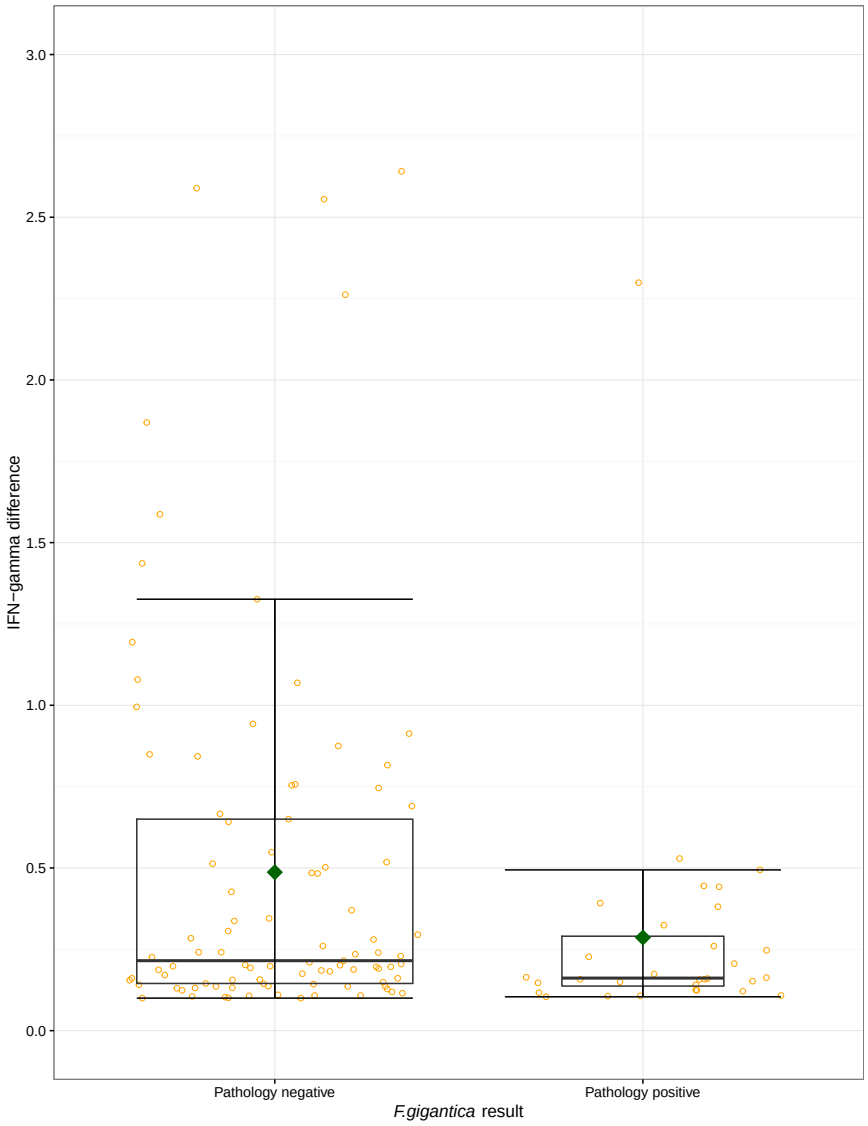


Figure 5.7: **Interferon-gamma response stratified by *F. gigantica* pathology status.** In a subset of *M. bovis* culture positive cattle (n=86). Key: Mean= Green diamond; Individual cattle; Orange circles.

5.4 Discussion

It is well established that *F. hepatica* infections are able to modulate their hosts immune responses to avoid elimination, characterised by up-regulation of Th2 and suppressed Th1 responses (286; 275; 284; 283). Such immune modulation negatively impacts on bTB diagnosis because current diagnostic tests are based on detection of Th1 immune responses (78; 48; 193). Comparatively little research has been undertaken on *F. gigantica* co-infection and the potential impact of its immune modulation on infections such as *M. bovis*. This chapter begins to build an evidence base that *F. gigantica* may also have affects on bTB pathogenesis and diagnosis (Figure 5.8). There is a strong association between *F. gigantica* infection and the occurrence of visible TB lesions in a naturally infected cattle population. Another population-based study in Zambia also looked at *M. bovis* and *F. gigantica* co-infection in slaughtered cattle and demonstrated a similar relationship (309). Development of CMI responses in immunocompetent cattle usually limit TB lesion expansion within the host (137). In the later chronic stages of bTB CMI immunity can wane, with IFN-γ responses decreasing with tubercle size and bacterial load potentially increasing with reduction in immunity (258). Host immune modulation, by *Fasciola* species, has been shown to be systemic rather than localised to the site of infection (98). Reduced CMI responses may lead to expansion of TB lesions from micro lesions to gross visible lesions elsewhere in the host. Although minimally investigated in cattle, evidence in a mouse experimental study that showed mice infected with *M. tuberculosis* have higher bacterial loads and TB lesion pathology when co-infected the trematode *S. mansoni* (296). As TB lesions were associated with *F. gigantica* co-infection in the abattoir study, it is possible that the parasite's immunosuppression of Th1 responses hastens the development of bTB pathology towards a disease state seen in cattle with chronic *M. bovis* infections.

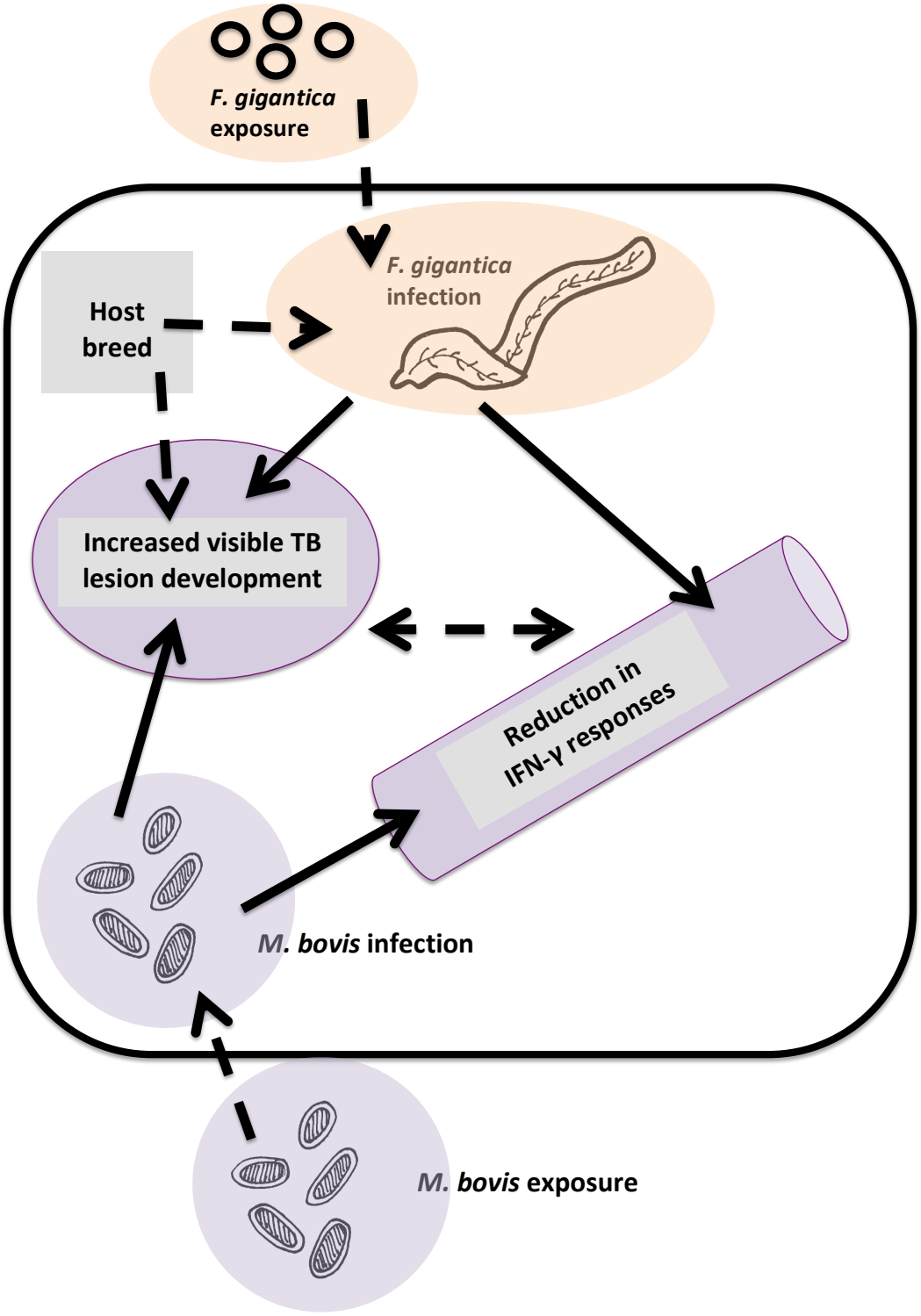


Figure 5.8: **A schematic diagram to summarise the results of co-infection relationship between bovine tuberculosis and *F. gigantica* explored in this study.**

Key: Bovine tuberculosis factors= purple; *Fasciola gigantica* factors= orange; Host factors= grey areas; Host= Area outlined in black; Interactions (Demonstrated and considered in this chapter)= Solid and dotted black arrows respectively.

A population based study investigating *M. tuberculosis* infection in people, showed that those co-infected with immunosuppressive HIV developed systemic TB with severer pathology compared to those not infected with with HIV (382). However in the abattoir study, TB lesions that were detected in co-infected cattle did not have a higher or more extensive lesion score (OLS) than cattle only infected with *M. bovis*. A recent laboratory study demonstrated that cattle infected with *M. bovis* and *F. hepatica* had lower IFN-γ responses, compared to those without *F. hepatica* co-infection, yet there was no difference in TB lesion size between the two groups (301). HIV infection in people might have a larger immunosuppressive effect on TB lesion development as the virus causes a more of a generalised immunosuppression through destruction of CD4+ T cells (393), compared to *Fasciola* species infections that only down-regulate Th1 immune responses. In another experimental study six 6-month old calves were co-infected with the same dose of *M. bovis* and *F. hepatica* and six with *M. bovis* only (298). Although not statistically significant, 14 weeks later at PME the co-infected calves had larger TB lesions than those infected with *M. bovis* alone. *Fasciola gigantica* burden is likely to fluctuate during the course of infection and thus the affect on TB lesion development might wax and wane throughout an animal's lifetime. In the abattoir study it was unknown how long cattle had been infected with either *M. bovis* or *F. gigantica*. Due to the design of the abattoir study it is impossible to account for changes in burden, which might affect extent of lesion development, and is a point for future research.

In addition, Fulani cattle were more likely to have TB lesions than mixed breed cattle. In Cameroon Fulani cattle have been noted previously to have a higher prevalence of bTB than other breeds (115). Yet there appears to be an interaction between *F. gigantica* status and breed. It has been noted that certain breeds of cattle include genotypes that have different susceptibility to bTB. In one population based study in

the UK, Holstein *B. taurus* cattle with the INRA111 genotype appeared to be less likely to develop bTB (394). Furthermore this had lead to development of a genetic index to grade Holstein susceptibility to bTB (395). In Ethiopia, Holstein cattle have been reported to have an higher prevalence of bTB compared to indigenous zebu *B. indicus* cattle (96). Although N'Dama *B. indicus* breed is known to be resistant to trypanosome infections (308), little work has been conducted on genetic resistance to bTB or *F. gigantica* in *B. indicus* cattle. It is also possible that Fulani cattle are managed differently to mixed breed cattle and have a higher exposure to *M. bovis* or *F. gigantica* than mixed breed cattle.

Fulani breed cattle may not be routinely treating for *Fasciola* or their grazing managed differently such that they have increased burdens. Due to the nature of the abattoir sample little is known how cattle were reared prior slaughter, such as grazing habits or frequency of anthelmintic treatments. Variation in cattle husbandry practices will be further explored in chapters 7 and 8) from population-based samples.

This study has also demonstrated for the first time, in a naturally infected cattle population, that IFN-γ responses were reduced with *F. gigantica* co-infection. In cattle Th1 responses are predominant in *M. bovis* infections (396) including IFN-γ responses. Similarly previous studies have demonstrated that *F. hepatica* co-infection can down-regulate IFN-γ responses to *M. bovis* infection (302; 298). The IFN-γ assay is particularly useful to detect *M. bovis* as early as 1-4 weeks post infection for accurate prevalence estimates or as part of control programs (161; 41). Using Bayesian estimation it was shown that suppressed IFN-γ responses, with *F. gigantica* co-infection, could result in a significant decline in the sensitivity of the IFN-γ assay. Hence where the IFN-γ assay is used to estimate prevalence, the decreased sensitivity with *Fasciola* co-infection may lead to underestimation of bTB prevalence. Also with the chronic nature of bTB, and difficulties in detecting *M. bovis* infection even at

PME, it is difficult to attain large sample sizes to investigate the complexities of the *M. bovis*/ *F. gigantica* co-infection dynamic. At minimum the results in this chapter encourage use of population data to investigate co-infection dynamics between *M. bovis* and *F. gigantica*. The potential impact of the relationship between IFN-γ responses and exposure to *F. gigantica* will be further explored using data from the cross-sectional study in chapter 8.

The results outlined in this chapter, support previous research on the effect of *Fasciola* species co-infections on *M. bovis* induced IFN-γ responses. Although a relationship between IFN-γ responses in *M. bovis* infected cattle and *F. gigantica* was identified in this study it is worth noting additional limitations of this research. The aim abattoir study was designed to investigate the prevalence of bTB in Cameroonian abattoirs as part of the CAMbTB larger project and not to investigate the co-infection relationship. Consequently the design of the study limited the size of the sample used in the co-infection analysis. For example cattle sampled from Bamenda abattoir were excluded from the analysis, due to the low prevalence of *F. gigantica* in Bamenda. If the abattoir study was designed to investigate the relationship between bTB and *F. gigantica* co-infection, sampling may have been conducted for longer in Ngaoundere abattoir. To increase the sample size and power of the study to investigate the co-infection relationship. Particulary for affect on TB lesion size and IFN-γ responses.

Presence of *F. gigantica* pathology implies evidence of previous or current infection rather than a direct measure of active infection. Evidence of *F. gigantica* pathology does not fully describe the complex relationship between the bTB and *F. gigantica* infections in cattle. It is unknown if slaughtered cattle within the abattoir study had previously been treated with anthelmintic to eliminate *F. gigantica* infection. Cattle also can become resistant to *F. gigantica* infections once their liver is sufficiently

damaged, such as calcification of the bile ducts, and possibly prevents adult parasites from feeding (210). Thus liver pathology, from previous *Fasciola* infections, may hinder future infections from completing their life cycle. Once *F. gigantica* infection is eliminated from cattle, it is unclear how long immune modulation responses persist. Fluctuations in burden or exposure to *F. gigantica* are likely to influence co-infection impact on Th1 immune responses and bTB pathology. It would have been useful to detect presence of current infection in slaughter cattle such as identification of *F. gigantica* parasites at meat inspection or using other diagnostics such as collecting faeces for FWECs. Longitudinal challenge studies, of *M. bovis* and current *F. gigantica* co-infection, could be useful to assess the the effect of changing parasite burden overtime.

Identification of *F. gigantica* infections in Cameroon is supported by previous studies in the country (215; 214) and in the surrounding Central-West African region (220; 397). Down-regulation of Th1 responses, by either *F. hepatica* or *F. gigantica* infection, is likely to have significant affects on bTB diagnosis using the IFN-γ assay. Despite the ecological, genetic and antigenic differences between *F. gigantica* and *F. hepatica* both species appear to evade and modulate the host immune response to infection (269). It is likely that *Fasciola* species have the similar immune modulatory mechanisms (198; 269), although the magnitude of down-regulation of Th1 responses may vary between *Fasciola* species infections. A 50-80% reduction in IFN-γ assay test sensitivity was reported in studies with *F. hepatica* co-infections (302; 298) and in this abattoir study a 20% reduction in test sensitivity was noted. Demonstrating that magnitude of co-infections effects may differ between *Fasciola* species.
Unfortunately due to the field-based nature of the abattoir sampling it was not possible to identify the species of *Fasciola* present in every animal. Presence of *F. hepatica*, mixed species or hybrid species infections could not be ruled out in Cameroon. Thus

variability in *Fasciola* species infection, could vary the magnitude of co-infection impact on bTB diagnosis and pathology across cattle sub-populations in Cameroon.

In summary, *F. gigantica* co-infections may impact bTB pathology and IFN-γ diagnostic sensitivity in this setting. It is important to note that although an association between bTB and *F. gigantica* co-infection was noted in this chapter; cause and effect were not proven. There is still much to be elucidated about the affect of *F. gigantica* co-infection on bTB diagnosis, with a fluctuating parasite burden, and on bTB pathogenesis overtime. However arguably in light of these results; the potential impact of exposure *F. gigantica* co-infection on bTB diagnosis should be explored when describing the epidemiology of bTB in Cameroon.

Chapter 6

Development of a Fasciola gigantica serum antibody ELISA

6.1 Introduction

Determination of bTB prevalence and identification of risk factors in Cameroon is dependent upon the ability of diagnostic test to detect bTB positive cattle. Using the IFN-γ assay to identify bTB positive cattle could be hindered by false negatives results (371; 370). Subsequently poor diagnostic test performance will impact on the reliability of bTB risk factor studies. Bovine tuberculosis positive cattle co-infected with *F. hepatica* species have been shown to be at greater risk of a false negative diagnosis using the IFN-γ assay (285). Risk factors for bTB were recently investigated in the UK using the SCITT for diagnosis (299). Multivariate logistic regression risk factor analysis was utilised to investigate risk factors. Presence of *F. hepatica* exposure, determined by a *F. hepatica* antibody ELISA, was included in the final risk factor model and improved model fit. *Fasciola gigantica* was shown to be present in Cameroon (Chapter 5) and to be a risk factor for false negatives for IFN-γ diagnosis of bTB (Chapters 4 and 5). Hence exposure of *F. gigantica* should be taken into account when investigating bTB risk factors in Cameroon. To be able to account for *F. gigantica* when investigating bTB risk factors, using IFN-γ positivity, an accurate diagnostic method needs to be used to diagnose *F. gigantica* exposure in live cattle.

Serological tests have been developed to detect exposure to *F. hepatica*. It is known that *F. gigantica* infections promote a humoral immune response (197). A variety of antigens have been used to develop serological diagnostics for use in ruminants and man (398; 226; 197). The majority of serological diagnostics use ELISA methods to detect humoral responses to ESP antigens secreted by the parasite such as crude ESP, purified products including cathepsins and f2 fragments (198; 239). As there are molecular and antigenic differences between *F. hepatica* and *F. gigantica* ESP

antigen-based humoral responses are thought to vary between these two species (269; 399). Therefore a species specific ELISA should be used depending on the species known to be present in the area. Although serological ELISA tests have been developed to detect exposure to *F. gigantica* ESP antigens in cattle (400; 235; 399; 247; 401) (Sensitivity; 81.8-100% and specificity; 91.6-98.9%) none are currently available commercially.

The aim of this chapter is to describe the development of a *F. gigantica* serological ELISA. The diagnostic performance of the ELISA is estimated and used to define a positive cut-off value. The effect of *F. gigantica* exposure on risk of cattle being bTB positive will be investigated in a chapter 8 of this thesis.

6.2 Materials and methods

6.2.1 Development of the *F. gigantica* antibody ELISA

ESP antigen

Two cattle slaughtered in Bamenda abattoir Cameroon had live *Fasciola* dissected from their livers to subsequently collect ESP antigen. In total 48 parasites were collected from cow AAA02101 and 34 from cow AAA03102. Methods used to collect ESP antigen post parasite culture are described in section 3.4.1 of this thesis. Excretory/ secretory antigen supernatants from 10 parasites per cow were pooled into two composites (ES1021 for cow AAA02101 and ES102 for cow AAA03102). The species of these parasites was confirmed using PCR (Chapter 5). The protein concentration of ESP antigen composites was measured using a total protein assay (Coomassie Plus (Bradford) assay, Thermo Scientific®). A protein concentration of 100-1500ug/ml was considered adequate for use in the ELISA protocol (246).

ELISA laboratory method

A protocol used for a *F. hepatica* antibody ELISA (246) was used to develop a *F. gigantica* antibody ELISA. The protocol was modified using collected ESP antigen from *F. gigantica* whole parasites. Control serum samples were used to determine appropriate ESP antigen and reagent concentrations to optimise diagnostic test efficacy (Subsection 3.4.1). An outline of the final method used for testing serum samples using the *F. gigantica* antibody ELISA and interpretation formulae are described in subsection 3.4.1 of this thesis.

Positive and negative controls

The positive control serum for the *F. gigantica* antibody ELISA was from a cow slaughtered at Bamenda abattoir, NWR, Cameroon with active infection identified at slaughter in the abattoir study (Animal ID: AAA03021). The negative control serum for the ELISA was from a cow that had been kept indoors all her life at Ness Heath farm at Leahurst Campus, University of Liverpool, Wirral, UK. The cow was only fed feed, including preserved forage, with no previous history of exposure *F. hepatica* metacercariae. Furthermore on annual diagnostic testing, including FWEC, copro-antigen and serum ELISAs, the cow was always diagnosed as negative for *F. hepatica* infection. Furthermore *F. gigantica* infections have never been reported in UK cattle.

6.2.2 Determination of a diagnostic cut-off for the ELISA

Positive and negative samples

Serum samples of positive and negative *F. gigantica* status cattle were tested, using the *F. gigantica* antibody ELISA, to calculate a positive diagnostic cut-off OD value for the ELISA. Positive and negative cattle were not primarily sampled for the validation of the *F. gigantica* antibody ELISA and the limitations of using these samples to define the accuracy of the ELISA are discussed in the section 6.4.

Fasciola gigantica positive cattle (n=20) were identified at meat inspection and cases were confirmed grossly by investigators (R. F. Kelly and S. Mazeri) in the abattoir study. There were 10 cattle from Bamenda abattoir and 10 cattle from Ngaoundere abattoir used in for the analysis conducted in this chapter. *Fasciola* pathology scores were recorded for these cattle (Section 3.4.2).

The *F. gigantica* negative sample (n=72) included cattle sampled in the UK and Cameroon. Due to differences in sampling methodology, the definition of known negative status differed for cattle sampled in the UK and Cameroon. Cattle sampled in the UK were kindly donated from a longitudinal abattoir study conducted in 2013-14 (n=15) (234). Cattle were identified to be *Fasciola* species negative by FWEC, detailed liver PME, *F. hepatica* serum antibody ELISA and *F. hepatica* copro-antigen ELISA (246; 239). Furthermore *F. gigantica* has never been diagnosed in UK cattle populations. To increase the sample size an additional 57 dairy cattle sampled in Cameroon, which had all been treated with anthelmintic in the past 12 months and kept housed during that time, were assumed for the purposes of this analysis to be *F. gigantica* negative. The anthelmintic type used to treat individual dairy cattle is unknown. The limitations of using these samples to define the accuracy of the ELISA are discussed in the section 6.4 and the dairy cattle sample is further described in chapter 7 of this thesis.

Additionally 5 *F. hepatica* positive cattle were tested using the *F. gigantica* serology ELISA to assess for cross-reactions. The cattle were sampled in the UK abattoir study and confirmed *F. hepatica* positive by FWEC, detailed liver PME, *F. hepatica* serum antibody ELISA and *F. hepatica* copro-antigen ELISA (234).

Statistical analysis

To describe the *F. gigantica* ELISA results, descriptive statistics, such as proportions and 95% confidence intervals, are calculated. Percentage agreement and Cohen's kappa statistic were used to quantify agreement between *F. gigantica* status and the *F. gigantica* ELISA at various positive cut-off values. For reference; Cohen's kappa statistic were interpreted as =1 (Perfect agreement), 0.81-1 (Almost perfect

agreement), 0.61-0.8 (Substantial agreement), 0.41-0.6 (Moderate agreement), 0.21-0.4 (Fair agreement), 0.01-0.2 (Poor agreement), $\leq$0 (No agreement) (352). Sensitivity and specificity, including 95% CI, were calculated to quantify the *F. gigantica* ELISA test performance at various positive cut-off OD values. When selecting a suitable positive cut-off OD value the aim was to balance both sensitivity and specificity of the *F. gigantica* ELISA. Analysis using a ROC curve allowed for the refinement of the positive cut-off value for the *F. gigantica* ELISA. The *F. gigantica* ELISA was deemed to be acceptable if the area under the curve (AUC) was $\geq$0.8. This implied the diagnostic test had the ability to discriminate between diseased and non-diseased animals (134). Finally at the selected positive cut-off value, positive and negative predictive values were calculated to estimate the probability of an individual animal having or not having exposure to *F. gigantica* at various prevalences. Further background information on the statistical methods used is outlined in chapter 3.

6.3 Results

6.3.1 Development of a *F. gigantica* antibody ELISA

F. gigantica ESP antigen

All 20 parasites used to produce the two composites, ES101 and ES102, were identified as *F. gigantica* by PCR (Chapter 5). Composite ES102 was selected to be used to develop the *F. gigantic* antibody ELISA (614ug/ml) as the protein concentration of composite ES101 was considered low (99ug/ml).

6.3.2 Determination of a positive cutoff OD value of *Fasciola gigantica* antibody ELISA

A total of 92 positive (n=20) and negative (n=72) *F. gigantica* cattle sera were tested using the ELISA. Using the results, of tested positive and negative cattle, a ROC curve was generated to select a positive cut-off value that achieved optimal sensitivity and specificity (Figure 6.1). A positive cut-off value of 12.8 PP achieved best compromise in sensitivity (85.0%; CI: 62.1-96.8%) and specificity (90.3%; CI: 81.0-96.0%). The calculated AUC (83.6%) and Cohen's kappa statistic (κ=0.70) also supported that at 12.8 PP the ELISA can distinguish between positive and negative cattle. Specificity with increasing the positive cut-off value (At 15.0 PP; 91.9%; CI: 82.7-96.9%) yet there is substantial decrease in sensitivity (At 15 PP; 75.0%; CI: 50.9-91.3%) (Table 6.1). Furthermore at the 15.0% PP positive cut-off value the lower CI approached 50.0% and the AUC is $\leq$0.8. Implying the the test could be no better than chance at 15.0% PP positive cut-off value. Hence to balance sensitivity and specificity the 12.8 PP positive cut-off value is used in the remainder this thesis.

Using the 12.8 PP positive cut-off value, the predictive values of the *F. gigantica* ELISA were calculated at various prevalences of *F. gigantica* exposure. At <50.0% prevalence the NPV is >90.0% and the PPV is <90.0%. At >50.0% prevalence the PPV is >90.0% and the NPV is <90.0%.

Raw percent positive (PP) values were plotted with the determined cut off value (Figure 6.2). Of the positive cattle 3/20 were classed as false negatives. Two of these cattle were sampled from Bamenda abattoir and 1 from Ngaoundere abattoir, Cameroon. The 2 from Bamenda abattoir had a *Fasciola* pathology score of 2 and the 1 from Ngaoundere had a pathology score of 1. There is no association between magnitude of PP and *Fasciola* pathology score of positive cattle (Figure 6.3). Furthermore of the additional 5 *F. hepatica* positive cattle tested, 4/5 tested positive using the *F. gigantica* ELISA.

Of the negative cattle 7/72 are classed as false positives. All 7 are from dairy cattle sampled in Cameroon that have been treated with anthelmintic. A *Fasciola* pathology score is not available for *F. gigantica* negative cattle as they were not slaughtered at the time of sampling.

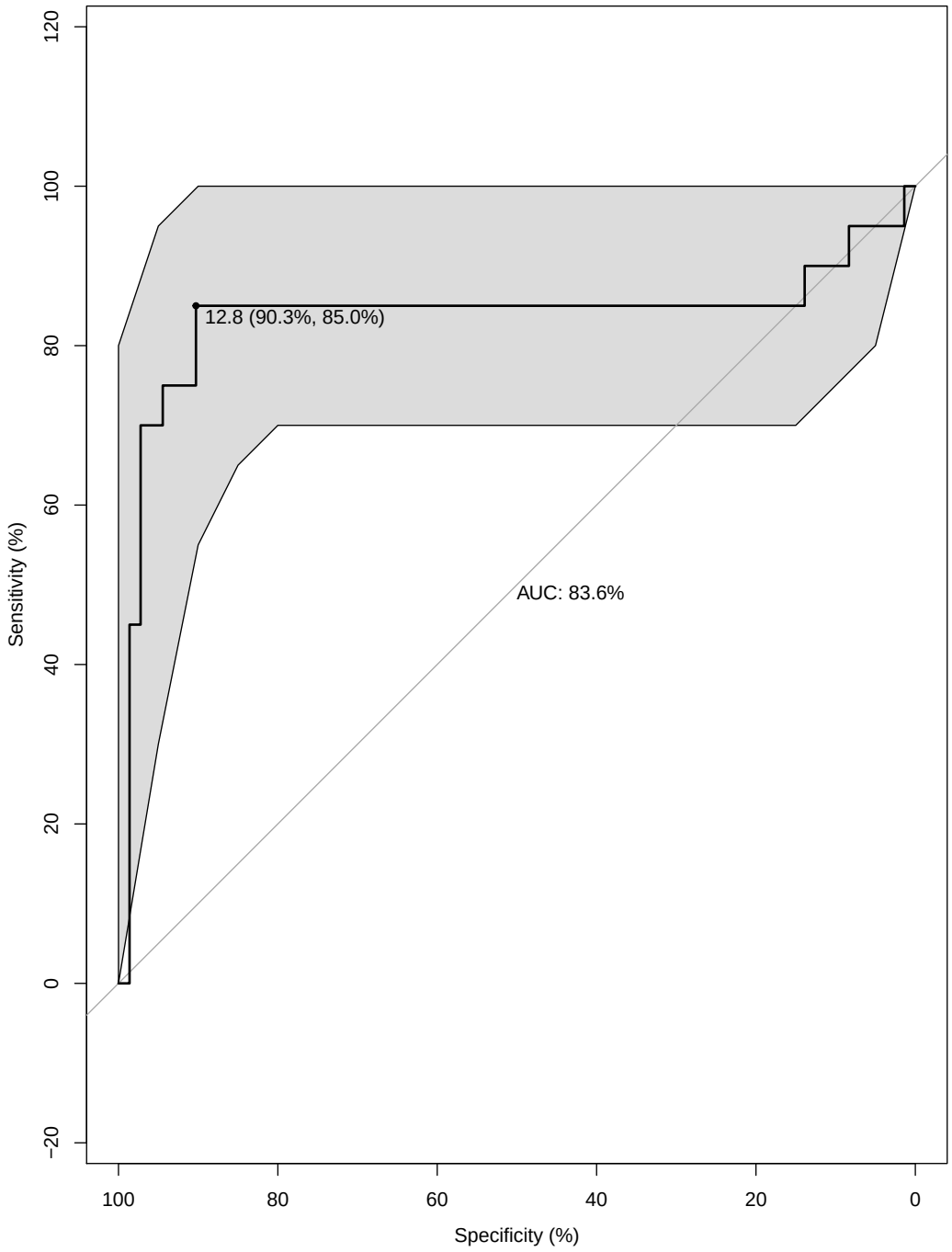


Figure 6.1: **The receiver operator curve (ROC) for the *F. gigantica* antibody ELISA of *F. gigantica* positive (n=20) and negative (n=72) cattle.**

The SE and SP of the ELISA is represented on the y and x axises respectively. The line ("Staircase trace") represents SE and SP, with 95% CI being the grey shaded area, at different positive cut-off values for the ELISA. A selected positive cut-off value of 12.8 PP balances sensitivity (85.0%) and specificity (90.3%) with an AUC of 83.6%.

***F. gigantica* antibody ELISA**	**Known *F.gigantica* status**			**Percentage agreement**	**Cohens kappa statistic (95% CI)**	**Sensitivity (95% CI)**	**Specificity (95% CI)**
10.0% PP		+	-	77.2%	0.47 (0.29-0.65)	85.0% (62.1-96.8%)	75.0% (63.4-84.5%)
	+	17	18				
	-	3	54				
12.8 PP		+	-	89.1%	0.70 (0.53-0.87)	85.0% (62.1-96.8%)	90.3% (81.0-96.0%)
	+	17	7				
	-	3	65				
15.0% PP		+	-	88.0%	0.65 (0.47-0.84)	75.0% (50.9-91.3%)	91.7% (82.7-96.9%)
	+	15	6				
	-	5	66				
20.0% PP		+	-	90.2%	0.70 (0.51-0.88)	70.0% (45.7-88.1%)	95.8% (88.3-99.1%)
	+	14	3				
	-	6	69				
25.0% PP		+	-	89.1%	0.64 (0.44-0.84)	60.0% (36.1-80.9%)	97.2% (90.3-99.7%)
	+	12	2				
	-	8	70				

Table 6.1: **Different possible positive cut-off values (PP) for the *F. gigantica* antigen ELISA with 2x2 table, percentage agreement, Cohen's kappa statistic, sensitivity and specificity. Selected positive cut-off value, used in the remainder of this thesis, is highlighted in grey.**

F. gigantica prevalence	Positive predictive value (PPV)	Negative predictive value (NPV)
1.0%	7.9%	99.8%
5.0%	30.1%	99.1%
10.0%	48.6%	98.2%
25.0%	73.9%	94.7%
50.0%	89.5%	85.7%
75.0%	96.2%	66.7%
90.0%	98.7%	40.0%
95.0%	99.4%	24.0%
99.0%	99.9%	5.7%

Table 6.2: **The positive and negative predictive values (PPV and NPV) of the *F. gigantica* ELISA at different prevalences. Positive cut-off value= 12.8 PP; Sensitivity= 85.0%; Specificity= 90.0%.**

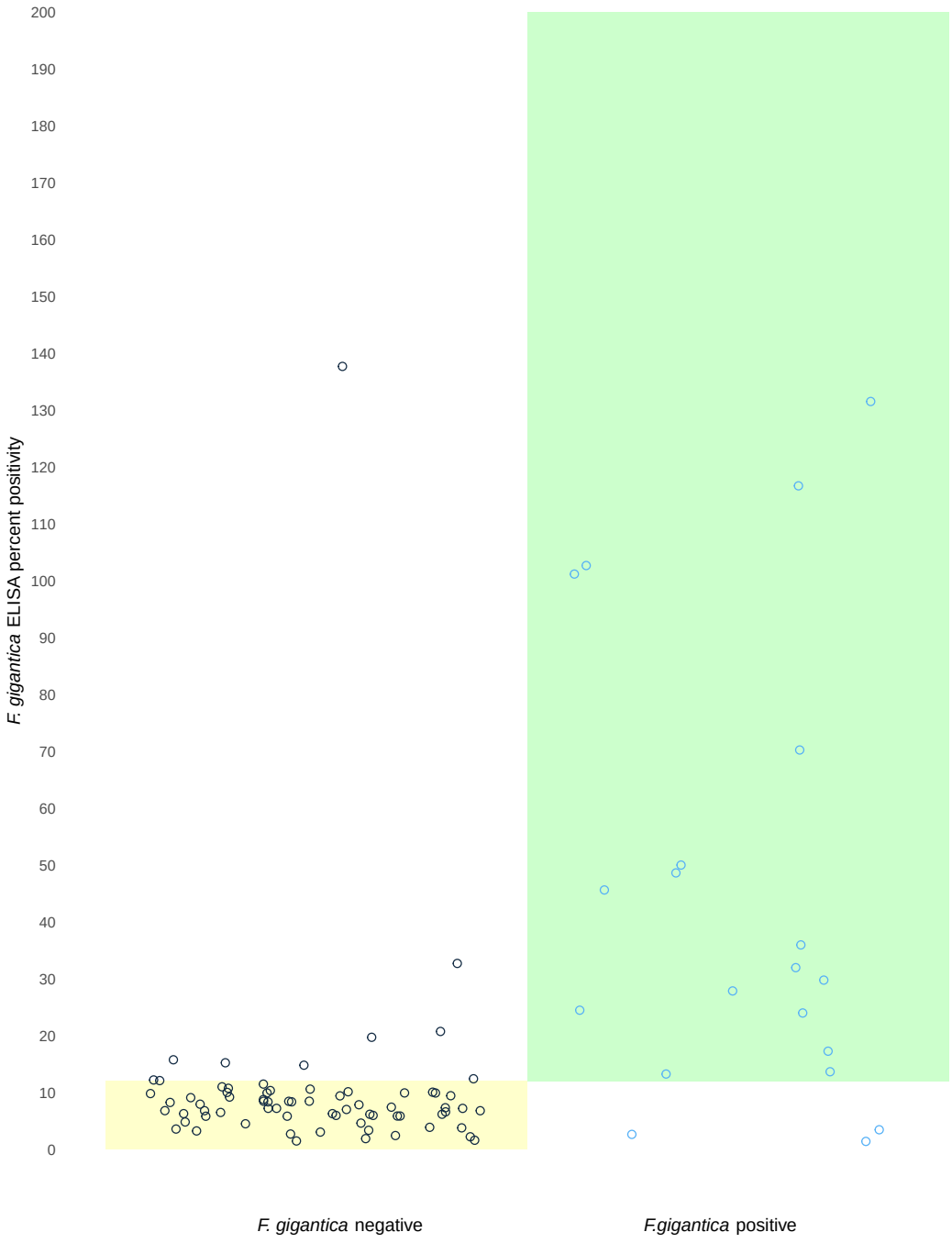


Figure 6.2: **Scatter plot of the *F. gigantica* antibody ELISA percent positivity (PP) values of *F. gigantica* positive (n=20, blue circles) and negative (n=72, black circles) populations.** For the *F. gigantica* antibody ELISA $\geq$12.8 PP is shown by the green area. The yellow area denotes test negative cattle for <12.8PP.

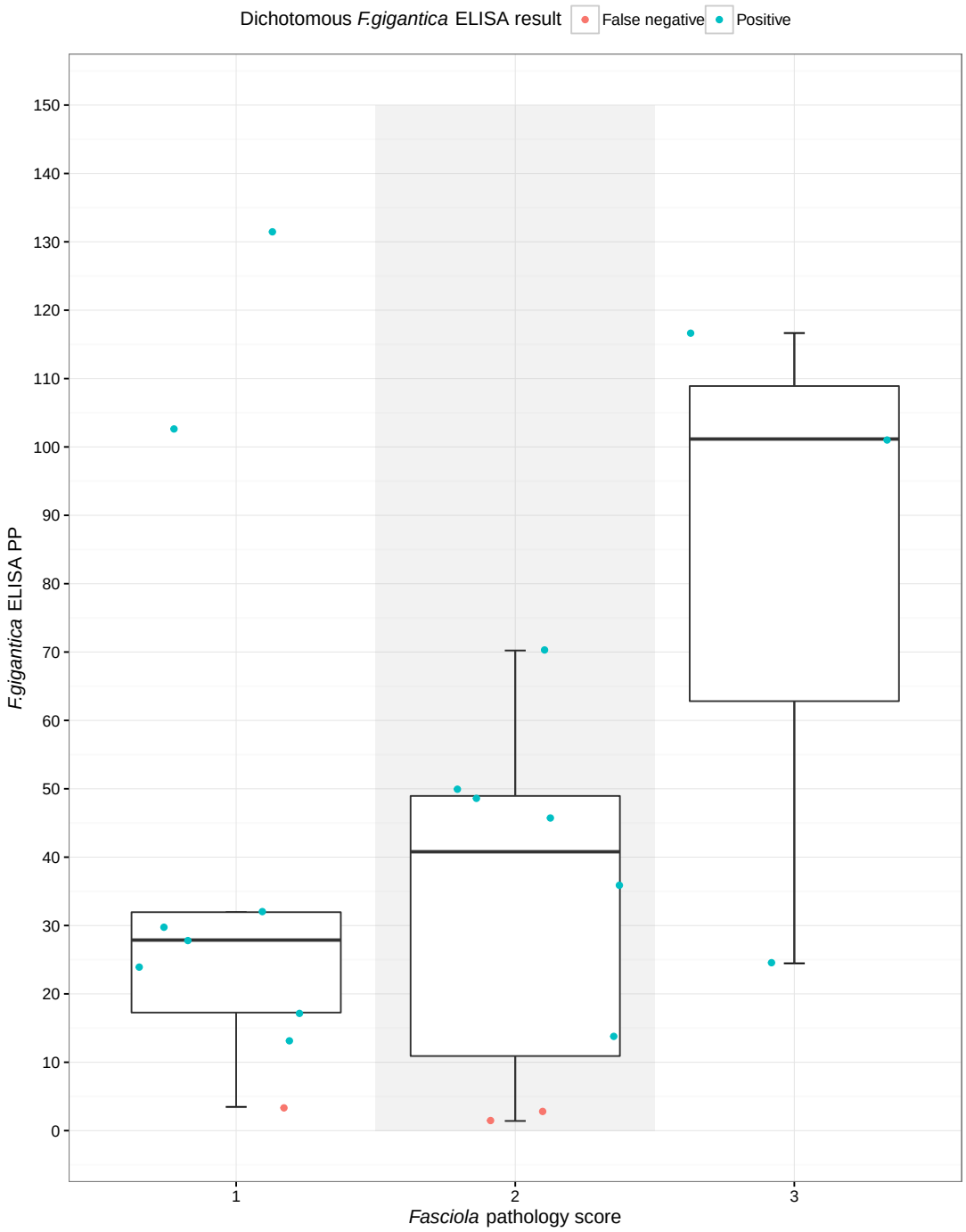


Figure 6.3: **Box plot of the percent positivity (PP) values of *F. gigantica* positive cattle (n=20) by *Fasciola* pathology score.**

Points represents the binary result of individual animals with positives detected as positive (blue) and false negatives (red) highlighted.

6.4 Discussion

The *F. gigantica* ELISA maintained 85.0% sensitivity (CI: 62.1-96.8%) and 90.3% specificity (CI: 81.0-96.0%) when selecting 12.8 PP as the positive cut-off value. Higher levels of specificity could be achieved, by increasing the positive cut-off value beyond 12.8 PP, but not without significant compromise in sensitivity. The reported sensitivity and specificity of the ELISA (12.8 PP) was within the range of other *F. gigantica* ESP antigen based ELISAs (Sensitivity: 81.8-100% and specificity: 91.6-98.9% (400; 235; 399; 247; 401)). Furthermore the sensitivity and specificity of the *F. gigantica* ELISA is comparable to the *F. hepatica* ELISA it was developed from (Sensitivity: 98.0% CI: 96.0-100% and specificity: 98.90% CI: 93.0-98.0% (246)). These findings are suggestive that, like the *F. hepatica* ELISA, the developed ELISA could potentially be useful for investigating *F. gigantica* effect on bTB diagnosis in Cameroonian cattle (299). However the less than perfect sensitivity and specificity, of the *F. gigantica* ELISA, is likely to be the result of suboptimal selection of positive and negative cattle in this study. It is important to consider the known limitations of the *F. gigantica* ELISA, and estimation of its performance (353), prior its use in further chapters.

A proportion of positive cattle were detected as negative regardless of the positive cut-off value used for the ELISA. Positive samples were only confirmed as positive by meat inspection rather than by detailed PME (Section 3.4.2) and therefore cattle sampled only had evidence of *F. gigantica* pathology. Implying that cattle only had previous exposure to *F. gigantica* at some point in their lives rather than current infection. Low *F. gigantica* burdens could have contributed to false negative cattle and resulted in a lower estimated sensitivity. The magnitude of ESP serological responses has been shown in a separate study to be semi-quantitative to burden of *F.*

gigantica in cattle (269) and cattle infected with low burdens of *F. hepatica* have been shown to have absent or lower ESP-induced IgG responses (239). Also the *F. gigantica* burden of positive cattle was unknown and will have likely fluctuated through their lives. IgG responses maybe influenced by fluctuations in total parasite burden overtime (402). However, responses are unlikely to significantly decrease overtime to produce a negative serological response if the parasite burden was initially high (239). The three false negatives identified, using the 12.8 PP cut-off value, had low *Fasciola* pathology scores (1-2). No association was noted between pathology score and ELISA PP value (Figure 6.3), this is likely because *Fasciola* pathology scores are not associated with total *Fasciola* parasite burden.

Species of *Fasciola* infecting positives cattle may have also affected the sensitivity estimate of the *F. gigantica* ELISA. Despite only *F. gigantica* species being identified in this study the presence of *F. hepatica* infections in Cameroonian cattle was not ruled out. The species of *Fasciola* was not determined for all *Fasciola* parasites infecting cattle in the positive sample. Furthermore, it was demonstrated that 4/5 *F. hepatica* infected cattle tested positive using the *F. gigantica* ELISA. It is possible that *F. hepatica* is present in Cameroon although this has been minimally investigated. Distribution of *Fasciola* species is dictated by the distribution of the intermediate snail host such as the aquatic snail species (e.g. *Radix natalensis* (403; 204)) and mud snails (e.g. *Galba truncatula*) for *F. hepatica*. Geographical distribution of *Fasciola* species across Africa is only partially understood but pockets of *F. hepatica* do occur in sub-Saharan Africa (205; 224) and intermediate host snail species could exist in Cameroon. Mixed burdens of *F. hepatica* and *F. gigantica* could have contributed to detection of both false negatives and positives in this study. There are ESP and tegumental protein similarities, between *F. gigantica* and *F. hepatica*, but also differences (197). In a study in Uganda by Howell and others used the *F. hepatica*

ESP antigen serological ELISA to detect exposure of cattle to *F. gigantica* and false negatives were also reported (250). Other studies have shown *F. hepatica* f2 antigens had 81.7% sensitivity and 100% specificity detecting *F. gigantica* infections in cattle and buffalos (222; 248; 249). Yet occurrence of cross-reactions may vary between specific antigens and populations of *Fasciola* which could affect apparent test sensitivity and specificity. In another study, mixed *F. gigantica* and *F. hepatica* infections, were used to calculate sensitivity and specificity of a *F. gigantica* based ELISA (237). The apparent sensitivity (86.0%) and specificity (70.0%) of the assay was much lower than other *F. gigantica* assays and could have been compromised in the presence of mixed *Fasciola* infections.

Selection of negative cattle is likely to have impacted on the reported specificity of the *F. gigantica* ELISA. At the selected 12.8 PP positive cut-off value 7/72 false positives were identified. The *F. gigantica* ELISA only detects exposure to *F. gigantica* infections, rather than current infection in cattle. Excretory-secretory proteins have shown to be detected in *F. hepatica* infections from two weeks post-infection while parasites are migrating through the liver (239; 404). Serological responses to *F. hepatica* have been reported to persist for at least six months post-infection for *F. gigantica* (269) and up to two years for *F. hepatica* (208). All seven false positives were from the negative dairy cattle sample from Cameroon (n=52). These cattle were assumed to be *Fasciola* negative as they were kept indoors and treated with anthelmintic in the previous 12 months. However their exposure to *Fasciola* species prior to this is unknown. Negative cattle could have been infected with *Fasciola* species previously and have persistent antibody responses despite effective anthelmintic treatment (269; 208). Hence the specificity of the *F. gigantica* ELISA maybe higher than reported due to the inadequate selection of cattle for the "known negative" sample.

Cross-reactions with other helminth infections could have contributed to false positives. Rumen flukes, such as *C. daubneyi* and *P. cervi*, have been reported to cross-react with *F. hepatica* ELISAs (405; 234). *Gastrothylax* species have been reported not to cross react with an *F. gigantica* serology somatic antigen ELISA (406) although it is unknown if they cross react with *Fasciola* ESP antigen serology ELISAs. Cattle sampled in the abattoir or cross-sectional studies were not tested for other co-infections. Hence cross-reactions with other parasites, particularly other trematodes such as schistosomes and rumen flukes present in Cameroon, were not ruled out in this study.

This is the first use of a serological assay to define *F. gigantica* status of cattle in Cameroon. The method defined provides a valuable method for others to use in future studies in Cameroon and other cattle populations where *F. gigantica* is present. The developed *F. gigantica* ELISA was used to investigate the effect of *F. gigantica* exposure on bTB diagnosis in Cameroonian cattle (Chapter 8) using the 12.8 PP positive cut-off value. However the reported sensitivity and specificity estimates are likely to be imprecise due to the positive and negative samples used to validate the ELISA. To improve the accuracy of the sensitivity and specificity estimates selecting better "known" positive and negative samples are required. Larger sample sizes would be useful to improve the accuracy of calculated estimates. Effect of size of *F. gigantica* burden and presence of other trematode co-infections on the performance of the *F. gigantica* ELISA should be investigated.

Chapter 7

Knowledge of Bovine Tuberculosis, Cattle Husbandry and Dairy Practices amongst Pastoralists and Small-Scale Dairy Farmers in Cameroon

Included as published article in PLOS One January 2016. The paper is introduced along with the results being summarised and discussed in the context of the thesis. The authors involvement with this article included the concept, all the analysis and being lead author of the manuscript:

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7.1 Introduction

In Cameroon, active epidemiological surveillance for bTB in cattle is limited to meat inspection in abattoirs to identify TB lesions. Using various of diagnostic tests, previous research studies have reported the prevalence of bTB in cattle up to 40% in Cameroon (77; 366; 114; 75; 120; 117; 115; 116). However the majority of these studies were conducted in abattoirs, in order to estimate prevalence of bTB in Cameroon's cattle population, yet it is unclear if the population of cattle slaughtered is truly representative of the country's cattle population to make such estimates. From a public health respect resale of milk is unregulated in Cameroon as veterinary/ public health infrastructure is limited (38; 407). A previous study in Cameroon showed awareness of zoonotic TB in cattle handlers in NWR Cameroon was high (67.9%) (408). Participants sampled in the study mainly worked in an abattoir where bTB meat inspection was conducted which was likely to raise awareness (120) hence awareness of bTB and extent of milk consumption remains unclear within cattle rearing communities.

Cameroon is an important cattle rearing country within Central Africa; exporting cattle to adjacent countries such as Nigeria, Gabon and Congo in addition to consuming much of the production itself. There are approximately six million cattle in Cameroon, mainly distributed over the higher mountains of the NWR, Adamawa and northern Regions of Cameroon (Chapter 2 Figure 2.1). Historically Cameroonian cattle production has been undertaken by the Fulani ethnic group, a pastoral community spanning Central and West Africa (332; 409). Cattle keeping is core to Fulani culture, not only, for meat and milk production but importantly as financial capital. They extensively graze *Bos indicus* cattle breeds and many still practice transhumance. This seasonal migration for grazing occurs in the dry season along

river valleys. Meat and milk are sold at cattle markets for local consumption. Live cattle are transported to the urban centres for national consumption and export. Over the past 20 years small-scale dairy farmer cooperatives have appeared in the NWR (42). These dairy farmers tend to be from non-Fulani backgrounds and rear small numbers of *Bos taurus* cattle, mainly Holstein-Frisian type animals, semi-intensively in basic stalled housing. Milk is sold through their farmer cooperative to peri-urban communities at local public markets. Insight into husbandry practices and bTB knowledge in these cattle rearing communities will aid identification of potential risk factors for bTB transmission in cattle populations (410; 122; 139). Understanding milk processing and consumption practices will provide further insight into the risk of zoonotic transmission in these communities (325; 408). Potentially influencing the future plans for bTB control in cattle and public health strategies in Cameroon

This published PLoS One paper reports on the findings from a cross-sectional study based on a structured questionnaire conducted in face to face interviews, with pastoralists and dairy farmers, in two study sites in Cameroon (Chapter 3). Cattle husbandry and dairying practices are described along with awareness of bovine infectious diseases in relation to bTB. The potential reasons for differences in bTB awareness within cattle rearing communities are investigated. The findings of the paper, in the context of this thesis, are subsequently discussed.

Suggested minor corrections to the paper:

1. Correction to sentence starting on line 13 of page 9/20 in the paper:
   "More pastoralists in the VD reported fasciolosis in their cattle at slaughter than pastoralists in the NWR and dairy farmers. Although more pastoralists in the NWR reported their cattle had been sick or died from fasciolosis than pastoralists in the VD and dairy farmers."

2. Correction to sentence starting on line 1 of page 14/20 in the paper:

   "The occurrence of pseudo-vertical transmission (E.G. Transmission of cow to calf via suckling of milk or close association via aerosol transmission) means that one infected herd may have given rise to a number of secondarily infected herds (56)."

7.2 Discussion

In cattle rearing communities, where bTB is present but control measures are minimal, quantifying bTB awareness is a valuable first step in assessing the need for disease control strategies. Understanding cattle husbandry practices may identify potential risk factors contributing to bTB transmission. Investigating local milk processing and consumption may highlight the risk of *M. bovis* zoonotic transmission. In Cameroon pastoralists and dairy farmers appear to be distinct cattle rearing populations with dissimilar husbandry and dairying practices. Diversity in husbandry practices was also noted between pastoralists in the two study sites. The results in this chapter outline the differences between cattle rearing communities that may influence local disease awareness, bTB transmission, and risk of zoonotic TB in Cameroon.

The majority of pastoralists were male, with no formal education and were from Fulani ethnic groups. Pastoralists rear cattle extensively at pasture and are commonly traded at markets. Pastoralists identified mainly as Mbororo and Fulbe Fulani sub-groups; who historically have kept cattle in Cameroon since the 17th century (332; 409). In other studies Cameroonian Fulani pastoralists were aware of other infectious diseases such as FMD and were able to recognise relevant clinical signs (315; 411). Awareness to FMD was high amongst pastoralists in this study and they could identify relevant clinical signs for FMD. Unlike pathognomonic diseases, like FMD, clinical signs for bTB are particularly variable and cattle with chronic pathology can present with few or no clinical signs (38; 412; 413). Various differentials for bTB are also present in this setting making identification of bTB difficult in this setting. Hence pastoralists and dairy farmers may have lower awareness of bTB if infected cattle are displaying no clinical signs of disease. But pastoralists who were aware of bTB, in both study sites, could identify relevant

clinical signs for bTB such as coughing and weight loss implying some experience of clinical disease.

Pastoralists in the NWR were more likely to be aware of bTB (OR: 6.27, CI: 1.41-27.87, $p<0.05$) than pastoralists in the VD. Increased prevalence of clinical bTB may increase bTB awareness through individuals recognising clinical signs of the disease. Hence higher bTB awareness in the NWR could be linked to higher prevalence than in the VD. Low bTB prevalence has been previously noted in the VD and linked to predominately Gudali cattle being kept in the VD (407; 117). Since the 1960s isolation of Gudali cattle has been undertaken, under Cameroonian law, in the VD and may have prevented mixing with infected cattle from outside the Division. Also nearly half of pastoralists in the NWR (43.8%) undertook transhumance compared to few in the VD (6.2%). The VD is highly suitable for grazing cattle being predominately savannah pasture with ample local provision of cotton seedcake; minimising the need for transhumance during the dry season (334). More pastoralists in the NWR identified as Mbororo ethnicity who culturally undertake transhumance compared to the Fulbe who are predominately reside in the VD. The majority of herds that undertook transhumance encountered more herds (5 or more other herds) than during normal grazing practices (1-5 other herds) and more pastoral herds in the NWR (74.8%) encountered other herds whilst watering than in the VD (45.7%). Increased contact with other herds has been shown to encourage the transmission of bTB (107; 413; 79). Additionally more herds in the NWR (54.7%) were fenced in together at night than in the VD (17.1%). Close contact between cattle may facilitate transmission by aerosol and faeco-oral routes from infected cattle (56; 57). Further research is required to understand contact networks of cattle in Cameroon, which might aid our understanding of *M. bovis* transmission in cattle.

Pastoral husbandry practices, common in both study sites, could be potential risk

factors for bTB transmission. Faceo-oral transmission of *M. bovis*, compared to aerosol transmission, is considered low when cattle are grazed at pasture. But the pathogen can survive in bovine faeces for at least two months and in soil for up to two years in damp conditions (62; 103). The rainy season in Cameroon may provide environmental conditions for *M. bovis* survival in contaminated faeces on pasture (38). African wildlife has been associated with high prevalence of *M.bovis* to cattle in Zambia and South Africa (414; 415; 105). Pastoral cattle grazing at pasture commonly have contact with wildlife species especially antelope. Species of antelope in Cameroon include eland (*Taurotragus* spp), roan antelope (*Hippotragus* spp), korrigum (*Damaliscus* spp), kob (*Kobus* spp) and duiker (*Cephalophus* spp) and are different species from South African studies (312; 416). In general cattle interaction with antelope and other wildlife is poorly defined and susceptibility of wildlife species to bTB in unknown (107). In Tanzania, where cattle bTB status is also often unknown, purchasing cattle from other herds increased the risk of bTB introduction (81).

In contrast dairy farmers predominantly rear a small number of *Bos taurus* Holstein-Friesian cattle in housed systems. In previous studies Holsteins kept in housed systems in Ethiopia and have been shown to have a higher bTB prevalence than *Bos indicus* breeds. Similar results have been shown in Cameroon (75). Cattle are mostly housed with no other herds and fewer dairy farmers trade cattle; management practices which may limit bTB transmission (57). Demographically dairy farmers differ from pastoralists with less cattle rearing experience and identifying as non-Fulani, Grassland ethnic groups. Nearly half of dairy farmers were female and at least had a primary education level. Awareness of bTB appears relatively high amongst dairy farmers (73.9%) with men being more aware of bTB (OR: 6.27, CI: 1.41-27.87, $p<0.05$) than women. In this study the interviewer was

male and in some societies women may be less likely to respond openly to men in an interview (417). However this usually concerns gender sensitive issues, such as abortion or sexuality, rather than gender neutral topics like livestock (418; 419). Female dairy farmers were also more likely to have lower awareness of FMD and fasciolosis implying a gender knowledge gap. In NWR Cameroon non-Fulani women have traditionally participated in subsidence crop agriculture; being detached from the male-orientated Fulani cattle rearing society (420). Land ownership conflicts have risen, between farmers and pastoralists, since the 1950s due to limited pasture with increasing crop agriculture, human populations and climate change (333; 421; 422). With rural women being increasingly encouraged into livestock production, as a route out of poverty, it is important to address the gender gap in livestock disease knowledge in this setting (420; 423; 424). Interestingly education level, ethnicity and length of time spent rearing cattle were not related to bTB awareness in either pastoralists or dairy farmers. Suggesting limited discussion and awareness of bTB in Cameroonian society generally. Furthermore dairy farmers appeared to have poor knowledge of the clinical signs of bTB with over half of dairy farmers (55.9%) unable to identify any. Suggesting limited understanding of clinical bTB. Independent of this study 4.3% of dairy farmers had had a SCITT previously conducted as part of a separate research programs on their cattle compared to none of the pastoralists. Having had the SCITT conducted may have increased their awareness of bTB, without knowledge of clinical signs for bTB, and potentially increasing awareness amongst dairy farmers in general through their dairy cooperatives.

In both pastoralist and dairy farmer's families there was widespread milk consumption. Milk processing techniques can eliminate *M. bovis* from milk to prevent zoonotic transmission; including souring or heating of fresh milk (27). Processing milk by souring, also termed acidification, was undertaken predominately

by pastoralists for their families. It is unclear whether acidification can destroy *M. bovis* via traditional milk souring techniques and there is minimal research in naturally infected soured milk (425; 426; 427). A study in Zambia shows milk spiked with *M. bovis* that is naturally soured does not consistently destroy the pathogen even when milk is soured for one to three days (428). Efficacy of acidification is partly dependent upon pH reached which varies with different souring techniques (429; 430). Culturally souring methods vary across sub-Saharan Africa and in Cameroon souring for one to two days, without a starter culture, has been previously reported (42). Similarly pastoralists in our study soured milk for a median of one day. It is unclear if the same techniques are used across Cameroon and if *M. bovis* is adequately destroyed to eliminate public health risk. Heating milk prior consumption, by pastoralistâĂŹs and dairy farmerâĂŹs families, is common. The risk of milk-borne zoonotic transmission can be mitigated by heating milk; but the process is temperature and time dependent (431). For example heating milk to 72° C for 15-60 seconds using industrial pasteurisation techniques or by heating milk at 63° C for 30 minutes can destroy *M. bovis* (27). Yet it is unknown whether milk reaches a sufficient temperature to destroy *M. bovis* or whether temperature is measured in Cameroon. It was a flaw in the questionnaire design that time along with temperature of milk was not recorded. Without this information it is unclear whether milk heating practices, by pastoralists or dairy farmers, are sufficient to provide protection against milk-borne transmission of *M. bovis*.

However dairy farmers, and to a lesser extent pastoralists particularly in the NWR, sell fresh-untreated milk to non-family members. It is unclear whom fresh-untreated milk is sold to and if these individuals process milk. As fresh-untreated milk processing is unregulated in Cameroon and awareness of milk borne diseases including zoonotic TB is low. Subsequently it is unlikely milk is processed primarily

to destroy milk borne pathogens like *M. bovis* and may pose a potential risk to public health (27). With intensive dairy farming been established in the NWR potential exposure to *M. bovis* may increase in the human population due to increased contact with cattle and availability of raw milk products. Additionally with increasing land pressures in the NWR may have lead to increased contact between pastoral cattle and increased transmission of *M. bovis*. In Nigeria individuals with pulmonary TB have been shown infected spoligotypes of *M. bovis* identified in Cameroonian cattle yet the route of transmission is unknown (8). As routine surveillance for bovine or zoonotic is not currently undertaken, the risk of *M. bovis* transmission to cattle or humans is unknown. Furthermore with pastoral and dairy cattle populations being managed in markedly different ways *M. bovis* transmission dynamics may differ in these cattle populations and the potential for zoonotic transmission greater with dairy farmers.

In many developed countries bTB has been controlled through sustained control strategies by understanding cattle production systems, local awareness of bTB and milk consumption practices (410; 122; 139). Differences in husbandry practices between dairy farmers and pastoralists, in the NWR Region and VD Division, may influence risk of bTB transmission in cattle populations. As bTB risk may not be homogenous between cattle populations assessment of bTB diagnostic test performance and potential risk factors is justified. Awareness of bTB is not consistent within pastoralists and dairy farmer communities. Awareness and clinical knowledge of bTB is particularly low in female dairy farmers and potentially identifies a gender divide in infectious disease knowledge. Targeted education programs may improve bTB awareness as part of a "One Health" control strategy for bTB. Milk consumption is high within pastoralist's and dairy farmer's families and resale of milk is commonplace in the NWR. Yet notably awareness of zoonotic TB was extremely low and efficacy of milk processing practices, to destroy *M. bovis*, is unknown. Processing

techniques in Cameroon are diverse and further research is required to understand if current techniques are effective in minimising zoonotic transmission of *M. bovis*.

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RESEARCH ARTICLE

Knowledge of Bovine Tuberculosis, Cattle Husbandry and Dairy Practices amongst Pastoralists and Small-Scale Dairy Farmers in Cameroon

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Abstract

Background

Control of bovine tuberculosis (bTB) and zoonotic tuberculosis (zTB) has relied upon surveillance and slaughter of infected cattle, milk pasteurisation and public health education. In Cameroon, like many other sub-Saharan African countries, there is limited understanding of current cattle husbandry or milk processing practices or livestock keepers awareness of bTB. This paper describes husbandry and milk processing practices within different Cameroonian cattle keeping communities and bTB awareness in comparison to other infectious diseases.

Study design

A population based cross-sectional sample of herdsmen and a questionnaire were used to gather data from pastoralists and dairy farmers in the North West Region and Vina Division of Cameroon.

Results

Pastoralists were predominately male Fulanis who had kept cattle for over a decade. Dairy farmers were non-Fulani and nearly half were female. Pastoralists went on transhumance

with their cattle and came into contact with other herds and potential wildlife reservoirs of bTB. Dairy farmers housed their cattle and had little contact with other herds or wildlife. Pastoralists were aware of bTB and other infectious diseases such as foot-and-mouth disease and fasciolosis. These pastoralists were also able to identify clinical signs of these diseases. A similar proportion of dairy farmers were aware of bTB but fewer were aware of foot-and-mouth and fasciolosis. In general, dairy farmers were unable to identify any clinical signs for any of these diseases. Importantly most pastoralists and dairy farmers were unaware that bTB could be transmitted to people by consuming milk.

Conclusions

Current cattle husbandry practices make the control of bTB in cattle challenging especially in mobile pastoralist herds. Routine test and slaughter control in dairy herds would be tractable but would have profound impact on dairy farmer livelihoods. Prevention of transmission in milk offers the best approach for human risk mitigation in Cameroon but requires strategies that improved risk awareness amongst producers and consumers.

Introduction

Mycobacterium bovis, a member of the *Mycobacterium tuberculosis* complex (MTC), is primarily an infection of cattle but also various domestic and wild animal species [1]. The pathogen is the cause of bovine tuberculosis (bTB) and in chronically infected cattle can be associated with poor health and production [2,3]. Zoonotic transmission, from cattle to humans, is of great concern with approximately 3% of all human tuberculosis cases being caused by *M. bovis* [4,5]. It is generally believed that zoonotic transmission occurs through close contact with infected cattle or through consumption of untreated milk. Hence in many high-income countries the control of bTB in cattle is primarily aimed to protect human health rather than animal health [6–8]. The zoonotic risk of food borne transmission has been mitigated through public health initiatives such as meat inspection and processing milk by heating to a high temperature [9]. Increasing awareness of bTB, through education programs, has also been integral to zoonotic tuberculosis (zTB) control [8,10]. Bovine tuberculosis eradication programs have also relied upon test and slaughter of infected animals due to the chronic nature of bTB, lack of treatments and effective vaccines in livestock populations [11]. Yet in many low-income countries, where the majority of zTB cases occur, few control measures are present despite the high prevalence of bTB in cattle and the potential risk to public health [4,5,12]. In the face of advances in human TB treatment and control; TB is still prevalent worldwide with 3.3 million cases annually reported by the World Health Organisation (WHO) with 81% of cases occurring in low-income countries. With agriculture being the main form of income in rural Sub-Saharan communities, with many living in close contact with their livestock and consuming fresh milk products, it is unsurprising that zTB is of concern [5,6,13]. In addition to increased animal protein consumption, including fresh milk, in many sub-Saharan African countries. As the goal of the WHO, in its "END-TB" program, is to eliminate all forms of human tuberculosis by 2035 it is paramount zTB is not overlooked.

The prevalence of bovine tuberculosis in sub-Saharan extensively managed herds, such as in pastoral systems, is often high but with low within herd prevalence [14,15]. In high income countries, in the presence of control strategies, bTB prevalence between herds is low but where

herds are infected the within herd prevalence is high. High within herd prevalence is often related to risk factors of intensive production systems involving housing cattle in close contact of one another [16–18]. Furthermore high bTB prevalence is also seen in intensive systems in sub-Saharan Africa [19]. Region-specific epidemiological data is often limited due to absence of cohesive disease surveillance systems [14,20,21]. It is likely that local practices will influence the variation in bTB prevalence and suggesting different local risk factors to *M*. *bovis* transmission[17]. Highlighting that understanding local cattle rearing systems is paramount prior to investigating bTB epidemiology. For example previous studies in Cameroon have reported bTB prevalence between 0.1–4.3% using lesion detection in abattoir based studies [22–24]. Higher prevalences have been reported using ante-mortem diagnostics (3.5–18.4%) such as the single comparative intradermal skin test and even higher using a serological assay high (37.2%) [25–27]. Furthermore the awareness of bTB and the extent of milk consumption within Cameroonian cattle keeping communities is poorly understood with only previous studies limited to butcher's knowledge of bTB [22]. Yet active epidemiological surveillance in cattle is limited, resale of milk is unregulated and bTB education campaigns are absent. Cameroon is an important cattle-producing country within the Central and West Africa region, exporting cattle to adjacent countries such as Nigeria, Gabon and Congo in addition to supplying meat and milk for national consumption [28]. There are approximately six million cattle in Cameroon, mainly distributed over the mountainous North West Region (NWR), the Adamawa plateau and more northern Regions of Cameroon. Historically, Cameroonian cattle production has been undertaken by the Fulani ethnic group, a pastoral community spanning Central and West Africa [29,30]. Cattle keeping is core to Fulani culture, not only for meat and milk production, but importantly as financial capital. The importance of cattle is further highlighted by the more than 70 words for "cow" in the local Fulfulde language [31]. The Fulani graze mainly *Bos indicus* cattle breeds on extensive communal pastures and many herdsmen still practice transhumance (seasonal migration) in the dry season (November until April) along river valleys to find pasture. A sophisticated network of markets, trade routes and abattoirs join the value chain from the production areas to the large urban centres that are major consumers of livestock products. Over the past 10 years small-scale dairy farmer cooperatives have appeared; particularly in the NWR Region [32,33]. These dairy farmers tend to be from non-Fulani ethnic groups without a long tradition of cattle keeping and rear small numbers of *Bos taurus* cattle, mainly Holstein-Friesian type animals, semi-intensively in basic stalled housing. Milk is sold through their farmer cooperatives to peri/urban communities at local markets. The high cost of surveillance and limited veterinary/ public health infrastructure in Cameroon, like many sub-Saharan African countries, means that bTB control is challenging and likely to require a holistic approach [34,35]. Insight into bTB knowledge and milk processing practices in cattle keeping communities will improve our understanding of the socio-anthropological context and inform the scope and need for veterinary public health policies/strategies in Cameroon [36,37]. Further understanding of differences in cattle husbandry practices could aid identification of risk factors and potentially improve bTB control within Cameroon and adjacent countries.

This paper describes cattle husbandry, milk handling practices, knowledge and awareness of tuberculosis within a population based sample of herdsmen and dairy farmers from North West Region and Vina Division in Cameroon. Bovine tuberculosis knowledge and awareness will be compared to other important infectious diseases, such as foot-and-mouth diseases (FMD) and fasciolosis, to assess general infectious disease awareness. Reasons for variation in bTB awareness will be explored between pastoralists and dairy farmers.

Materials and Methods

Study sites

The study sites were the North West Region (NWR) and Vina Division (VD) of the Adamawa Region of Cameroon. Both are of similar geographical size of ~17,000km^2 (Fig 1). The NWR is an anglophone region situated in fertile mountainous highlands, 500-3000m above sea level. Bamenda, the capital, is Cameroon's third largest city. The Region is densely populated (1,804,695 people) and an estimated 506,548 cattle are grazed there [38,39]. The VD is part of

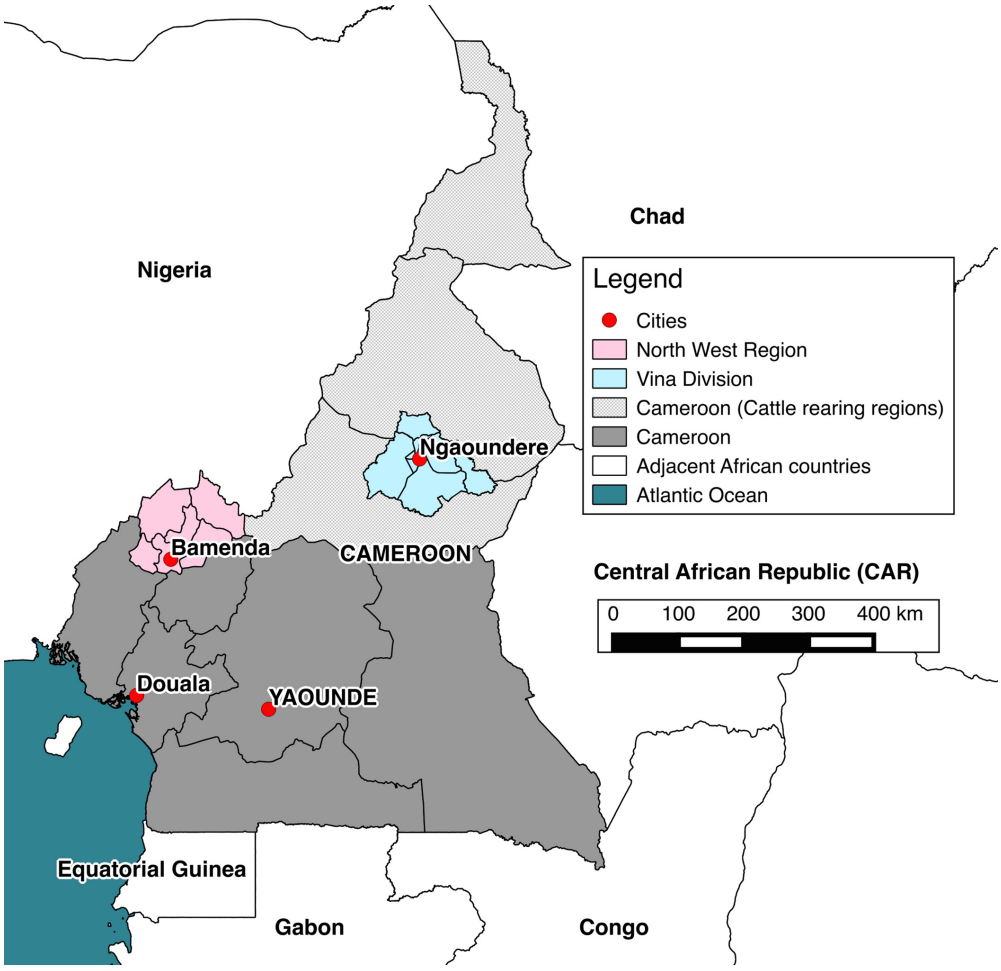

Fig 1. Map of Cameroon. The location of cattle rearing areas (light grey), study sites (pink and blue) and major cities (red).

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the fertile Adamawa Region's savannah plateau. The regional capital is Ngaoundere and the mainly francophone population of the VD (317,888 people) is much smaller than that of the NWR. The cattle population is also smaller with an estimated 176,257 head [40].

Veterinary services are predominately provided by the government through the Ministry of Livestock, Fisheries and Industrial Agriculture /Ministere de l'Elevage des Peches et Industries Animales (MINEPIA), with local veterinary technicians stationed at Zootechnical and Veterinary Sanitary Control Centres (ZVSCC) distributed across the country [41]. Their responsibilities include registration of local livestock keepers, disease control mainly through annual vaccination campaigns, meat inspection and regulation of livestock markets and animal movements.

Study design

Please note that we include brief mention of some details related to animal sampling for completeness so the reader can appreciate the context in which the herdsmen and farmer surveys were conducted but we will not present animal level data here as this analysis will focus only on the herdsmen and farmer knowledge and attitudes. Two cross sectional surveys were conducted between January–May 2013 in the NWR and September-November 2013 in the VD. The first was of pastoralists whose herds were listed in the Ministry of Livestock, Fisheries and Animal Industries vaccination records at 81 ZVSCCs in the NWR and 31 ZVSCCs in the VD in 2012. A total of 5,053 pastoralist herds in the NWR and 1,927 in the VD, with a range of 1–215 cattle per herd were included in the sampling frame. The list of herds in each site was stratified by administrative area; seven Divisions in the NWR and eight sub-Divisions within the VD and a random sample of herds was taken from each site proportional to the total number herds listed in each strata. This survey was part of a larger study of bTB and liver fluke and the sample size was based on a clustered random sample of cattle assuming a cattle level prevalence of ~10%, a within herd variance of 0.15 and between herd variance of 0.01, an average herd size of 70, a relative cost of 12:1 for herd:cattle and relative error of ±15% (Survey Toolbox; AusVet) [42]. This gave a target sample size of 15 cattle per herd and 88 herds under the simplifying assumption of perfect test performance. To allow for potential losses or drop out and to have balanced samples from the 2 sites, we aimed for 50 herds each of the NWR and VD and therefore herdsmen to be interviewed. Hence note that within the NWR or VD the estimates are unbiased but overall estimates require weighting to adjust for the different sampling intensities in the NWR and VD.

The second survey was of the small-scale dairy farmers who were all registered with Ministry of Livestock, Fisheries and Animal Industries and the address list for 2012 was obtained from their NWR office in Bamenda. Dairy co-operatives were established as part of a non-governmental organisation (NGO) initiative in the 1990s to improve milk production in the area. Donated Holstein-Friesian cattle from Ireland and Kenya were imported and given to families to be reared in zero grazing systems [33]. Calves born from these original cattle were then passed on to other members joining the cooperatives. There were 229 dairy farmers, grouped into 13 cooperatives with 3 to 52 farmers per cooperative. The cooperatives were categorised geographically into 4 groups. Three were spatially clustered with three cooperatives in each group. The fourth group consisted of four widely dispersed cooperatives and was not sampled for logistical reasons. Thus 164 dairy farmers were included in the sample frame. A stratified random sample of dairy farmers was selected proportional to the number of dairy farmers in each group. Again the survey was part of a wider study of bTB and fasciolosis and sampling cattle but based on the assumption of perfect test performance, a prevalence of ~6% in adult cattle (Nkongho et al, in press) and each dairy farmer having two adult cows resulting in a sample size of 46 dairy farmers.

Selected pastoralists and dairy farmers were contacted by phone or in person by the head of the local ZVSCC, and asked if they were prepared to participate in the study. Individuals were replaced by resampling if they declined, had died, moved out of the region, or were located more than three hours walk from a point that could be accessed by off-road vehicle, motorbike or on horseback.

Data collection

Pastoralist herdsmen were visited either at their homestead or at a convenient location in the vicinity where the cattle could be examined. Dairy herds were visited at the homestead. The translator/research assistant (SH) explained the project in either Fulfulde, Pidgin, English or French and the herdsman or farmer was asked to give verbal consent to participating in the study. It was made clear that this included completing a questionnaire about the management of their cattle and their knowledge of bTB, allowing the single cervical intradermal skin test (SCITT) to be conducted and a blood sample to be taken from their cattle for further diagnostic tests such as the bTB Y-interferon assay (Bovigam®) [3,43].

A structured questionnaire (S1 File) was administered by interview (SH) in the respondents preferred language. The questionnaire was developed through discussions with pastoralists, veterinary professionals and researchers. The questionnaire was pretested and modified prior to final use. The questionnaire took 20–30 minutes to administer. Questions asked focused on herdsman background, dairy practices routine herd practices, herd reproduction, grazing/housing, transhumance, cattle trade, and infectious diseases. Specifically knowledge and awareness of bTB was investigated but also FMD and fasciolosis as comparisons. Local names were used where appropriate such "Soharu", "Balki" and "Njobu" were used in Fulfulde and "Tuberculose bovine", "Douve du foie" and "Fièvre aphteuse" in French. Cameroonian Pidgin names for these diseases are the same as in English. Awareness of an infectious disease was defined as "the participant recognising the name of the disease". If pastoralists or dairy farmers were not aware of a particular infectious disease no more questions were asked relating to that disease. GPS location (Garmin eTrex® Venture) was also recorded. The questionnaire data were initially recorded in paper format and then transferred to a relational Access database (Microsoft Access®).

Statistical analysis

Samples sizes relate to the number of pastoralists and dairy farmers sampled. Statistical analyses were performed using packages and functions in R Studio 0.98® [44]. For pastoralists the study design was incorporated using the *svydesign* function in the *survey* package [45]. Descriptive statistics were estimated using *svymean*, *confint* and *svyby* functions to account for the design effects. Graphics were produced using the *ggplot2* package [46]. Maps were drawn using QGIS 2.2® [47] and shape files obtained from the open access GADM database of Global Administrative Areas (www.gadm.org). The multivariable mixed logistic regression models were developed using the R package *stats* and *glm* functions [48]. The main outcome variable was the dichotomous answer to the question "Are you aware of a disease called "Bovine Tuberculosis?". Similar questions were asked for fasciolosis and FMD. Explanatory variables were categorized as appropriate eg. "Location" was catagorized as strata (NWR and VD) for pastoralists and cooperative group for dairy farmers. A backwards stepwise approach was used to find the best fitting model to describe the dataset [49]. Model selection was based on the Akaike information criterion (AIC) and the best model was selected using the lowest AIC. Final model selection was verified by computing AICc and ΔAIC using the R package *AICcmodavg* and

modavg functions [50]. The p value, odds ratio with 95% CI for explanatory variables were also calculated.

Ethics statement

The study design and sampling methodology was reviewed and approved by the University of Edinburgh Ethics Committee, UK (ERC No: OS02-13) and by the Institute of Research and Development (IRAD), Cameroon. IRAD gave permission to conduct the fieldwork and issued fieldwork permits. The research did not involve endangered or protected species and no further approvals were necessary to conduct fieldwork. All participants gave informed verbal consent to participate and were aware they could opt out at any stage. Verbal consent was deemed appropriate for the variety of dialects spoken, variable literacy amongst participants and due to the remote outdoor fieldwork environment [41,51]. Information to be provided to participants, for informed verbal consent, was communicated to the interviewer (SH) in a written document. Additional training was provided to the interviewer regarding the consent procedure and interview process. Furthermore the interviewer was experienced in conducting questionnaires in similar studies and spoke the various local dialects of study participants [41]. Verbal consent was recorded on a cover sheet to the questionnaire by the interviewer and refusals were recorded in separate document along with reasons for refusal.

Results

In total 100 pastoralists were interviewed; 50 in the NWR and 50 in the VD. Of the selected herdsmen 23 were unavailable and these were replaced by randomly resampling from within the same ZVSCC list. Reasons for replacement included moving away from the study area (n = 4); had <10 cattle (n = 4), no longer kept cattle (n = 3); logistical issues (n = 6); herdsman name selected not known (n = 3); declined to participate at interview stage (n = 2) and the herdsman had died and their herd dispersed (n = 1). All 46 selected dairy farmers participated and none were replaced.

Participants, cattle and husbandry practices

Overall, 97.8% (CI: 86.4–99.7%) of interviewed pastoralists in the NWR and 100% (CI: 92.9–100%) in the VD were male. In contrast 43.5% (CI: 29.0–58.0%) of dairy farmers were female. There were differences in their formal schooling; 63.2% (CI: 50.0–74.7%) of NWR pastoralists and 74.0% (CI: 60.6–84.2%) of those in the VD had no formal schooling whereas all dairy farmers had some form of schooling, 76.1% (CI: 63.3–88.5%) at “primary school” level. The majority of pastoralists identified themselves as members of a Fulani ethnic group. In the NWR; 89.4% (CI: 77.4–95.4%) were "Mbororo" and only 2.0% (CI: 0.2–12.7) “Fulbe’; while in the VD 66.1% (CI: 53.0–77.1%) considered themselves as "Fulbe" and 17.6% (CI: 9.9–29.5) as “Mbororo”. The remainder were non-Fulani ethnic groups. None of the dairy farmers considered themselves to be from either Fulani group. Pastoralists in the NWR had worked with cattle for longer (26.5 years, CI: 22.4–30.5 years) than those in the VD (17.7 years, CI: 13.7–21.4). This was considerably longer than dairy farmers (5.5 years, CI: 4.0–6.9). Yet the mean ages of pastoralists in the NWR (41.0 years, CI: 37.0–44.9), VD (39.2 years, CI: 35.3–43.4) and dairy farmers were similar (45.8 years, CI: 42.4–49.3).

Reported pastoral herd sizes were larger in the NWR (50, CI: 45–55) than in the VD (38, CI: 34–43). Both were much larger than dairy herds (3, CI: 2–3). All pastoral cattle were *Bos indicus* or *Bos inducus/ Bos taurus* cross-breeds. Mixed breed (63.9%, CI: 54.6–72.2%) and red (16.1%, CI: 10.1–24.7%) and white Fulani (20.0%, CI: 12.9–29.8%) were mainly kept by NWR.

In the VD Gudali cattle (83.5%, CI: 78.1–88.8%) were the most common breed. Almost all dairy farmers kept *Bos taurus* Holstein-Friesian cattle (98.3%, CI: 95.1–100%).

All pastoralists (CI: 92.9–100%), in the NWR and VD managed their cattle extensively and 97.9% (CI: 86.6–99.7%) used streams as a source of drinking water. Almost all the pastoralists in both study sites contacted other herds during grazing (94.0%, CI:88.8–99.3%) and 67.0% (CI: 58.2–75.9%) had contact during watering; contacting one to 15 herds on a daily basis. Keeping cattle in fenced enclosures overnight was more common in the NWR (54.7%, CI: 43.1–66.3%) than in the VD (17.1%, CI: 8.8–25.3%). In contrast, 97.8% (CI: 93.6–100%) of dairy farmers housed their cattle and all (CI: 92.3–100%) used water troughs. Consequently, only 8.7% (CI: 0.5–16.9%) of dairy herds had contact with other herds.

There were regional and ethnic differences in the practice of transhumance. A greater proportion of NWR pastoralists (43.8% CI: 31.4–57.1%) undertook transhumance compared to with those in the VD (6.2% CI: 2.0–17.7%). Furthermore, across both pastoralist communities, 44.0% (CI: 30.1–58.0%) of Mbororo herdsmen went on transhumance compared with 10.7% (CI: 0–25.2%) of Fulbe. During transhumance, all herds came into contact with one to 15 herds on a daily basis.

When asked about contact with potential wildlife reservoir hosts, antelope were the most frequently contacted species. During normal grazing fewer herds in the NWR (49.8%, CI: 36.0–63.6%), reported antelope contact than in the VD (76.4%, CI:65.4–84.7%). During transhumance 80.9%, (CI: 64.5–97.3%) n = 25) reported antelope contact. Contact with buffalo was only reported during transhumance; it involved 25.4% herds (CI: 8.9–41.8%). Warthog contact was reported by 11.9% of herdsmen in NWR (CI: 5.4–24.0%) and 38.2% in the VD (CI:27.2–50.6%) when grazing and by 32.9% (CI:15.1–50.7%) during transhumance. All dairy farmers reported that none of the cattle came into contact with wildlife.

Natural service was used for breeding by all pastoralists (CI: 92.9–100%) and 89.1% (CI: 80.0–98.2%) of dairy farmers. In addition, artificial insemination (AI), using *Bos taurus* semen, was used by 10.2% (CI: 4.4–22.0%) of NWR pastoralists, 2.0% (CI: 0.0–5.8%) of VD pastoralists and 8.0% (CI: 1.7–14.4%) dairy farmers. Cattle selling was reported by 93.8% (CI: 83.2–97.9%) and 83.9% (CI: 71.3–91.6%) of pastoralists, in the NWR and VD respectively. A smaller proportion of pastoralists reported buying cattle in both the NWR (41.8%, CI: 30.0–54.7%) and VD (49.7%, CI: 36.4–62.9%). Most pastoralists in the NWR (83.4%, CI: 70.0–91.5%, n = 42) and VD (87.8%, CI: 75.4–94.4%, n = 44) traded at markets. Comparatively few dairy farmers sold cattle (37.0%, CI: 22.9–51.1%), purchased cattle (8.7%, CI: 0.4–16.9%) or traded at markets (11.8%, CI: 0.0–27.6%,).

When asked about treatments, anthelmintics (Albendazole or Ivermectin) use was reported by 100% (CI: 92.3–100%) of dairy farmers, 93.9% (CI: 82.6–98.1%) of pastoralists in the NWR and 84.2% (CI: 71.2–92.0) of those in the VD. Trypanosomiasis treatment was used by 77.7% (CI: 65.9–86.2%) of pastoralists in the VD compared with fewer 41.9% (CI: 29.1–56.0%) in the NWR. No dairy farmers treated for trypanosomiasis (CI: 0.0–8.6%).

Of the other susceptible species kept, goats were reported by about a third of pastoralists and dairy farmers; NWR (29.9%,CI: 19.2–43.3), VD (27.2%,CI:16.7–27.2), Dairy (30.4%, CI:20.9–48.7) and sheep by 45.2% (CI: 33.7–57.1), 28.8% (CI: 18.7–41.6) and 23.9% (CI: 11.5–36.4) of herdsmen respectively. Poultry were the most common species kept and were reported by 75.4% (CI: 61.2–85.7%), pastoralists in the NWR 65.4%, (CI: 51.1–77.4%) in VD and by 63.0%, (CI: 48.9–77.1%). dairy farmers. Horses were kept by pastoralists in the NWR (39.9%, CI: 28.2–53.0%) but were rarely kept by those in the VD (2.0%, CI: 0.3–12.4%) or by dairy farmers (2.2%, CI: 0.0–6.4%).

Knowledge of bovine tuberculosis compared to other infectious diseases

More dairy farmers (73.9%, CI: 61.1–86.7%) and NWR pastoralists (76.6%, CI: 63.4–86.1%) were "aware" of bTB than VD pastoralists (40.8%, CI: 30.1–52.5%). Nearly a quarter of herdsmen in the NWR reported cattle having died from tuberculosis or been informed about it from slaughter cases compared to <10% of those from the VD (Table 1). In contrast 4.3% (CI: 0.0–10.3%, n = 34) of dairy farmers had previously had a bTB SCITT conducted within their herd and an animal reported positive. The proportion of pastoralists that could not identify clinical signs for bTB was 18.4% in the NWR (CI: 5.9–30.9%, n = 38) and 23.8% (CI: 5.1–42.5%, n = 21) in the VD. But over half of dairy farmers (55.9%, CI: 38.9–72.8%, n = 34), who were aware of bTB, could not identify any clinical signs for bTB. Pastoralists, who were aware of bTB, identified coughing, weight loss, poor coat and weakness as signs of bTB and the pattern for the NWR and VD are almost identical (Fig 2).

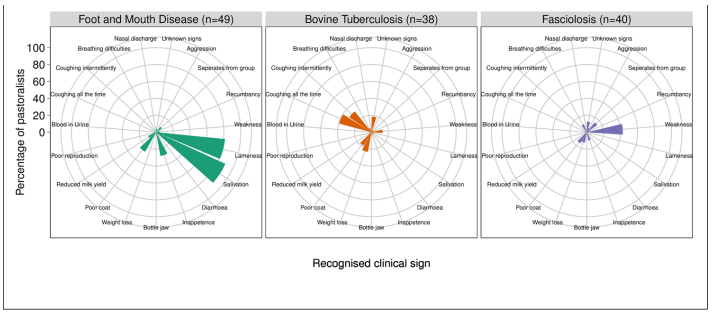
More pastoralists in the NWR (80.1%, CI: 68.1–88.3%) and VD (89.9%, CI: 78.4–95.6%) were aware of fasciolosis than dairy farmers (21.7%, CI: 9.7–33.8%). More pastoralists in the VD reported fasciolosis in their cattle than pastoralists in the NWR and dairy farmers (Table 1). In the NWR weakness was the most frequently reported clinical sign for fasciolosis (42.5%, CI: 27.0–58.0%, n = 40). Other clinical signs reported, by NWR pastoralists, were poor coat, bottle jaw, inappetence, separating from the group, nasal discharge and breathing difficulties. Weight loss (82.2%, CI: 70.9–93.5%, n = 45) was the most frequently reported by VD pastoralists along with poor coat, bottle jaw, inappetence and weakness (Fig 2). Only 12.5%, (CI: 2.1–22.9%, n = 40) of pastoralists in the NWR and 2.2% (CI 0.0–6.6%, n = 45) in the VD were unable to identify any clinical signs for fasciolosis when compared to the majority of dairy farmers (80.0%, CI: 53.9–100%, n = 10).

In comparison more pastoralists in the NWR (97.9%, CI: 86.6–99.7%) and VD (96.0, CI: 85.4–99.0%) were "aware" of FMD than dairy farmers (56.5%, CI: 42.0–71.0%). All pastoralists could identify at least one clinical sign of FMD whereas 23.1% (CI: 6.5–39.6%, n = 26) of the dairy farmers were unable to identify any clinical signs of FMD. Pastoralists frequently

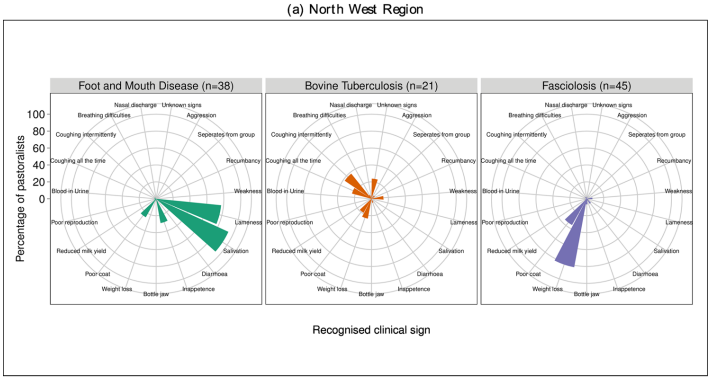
Table 1. Proportion of herds reported to have had various infectious diseases based on the subset of pastoralist and dairy farmers who reported being aware of the given infectious disease.

	North West Region Pastoralists (95% CI)	Vina Division Pastoralists (95% CI)	North West Region Dairy Farmers (95% CI)
BOVINE TUBERCULOSIS (bTB)	(n = 38)	(n = 21)	(n = 34)
Have you been informed of any cattle sold or slaughtered have bTB?			
Yes	23.5% (13.1–38.5%)	8.5% (2.1–27.8%)	0.0% (0.0–7.7%)
FASCIOLOSIS	(n = 40)	(n = 45)	(n = 10)
Have any of the cattle presented been sick from fasciolosis?			
Yes	47.5% (32.3–63.3%)	34.4% (22.4–48.9%)	0.0% (0.0–7.7%)
Have any of your cattle died from fasciolosis?			
Yes	35.2% (21.6–51.8%)	10.7% (4.5–23.4%)	0.0% (0.0–7.7%)
Have you been informed of any cattle sold or slaughtered have fasciolosis?			
Yes	15.2% (6.9–30.1%)	28.3% (17.1–43.0%)	0.0% (0.0–7.7%)
FOOT AND MOUTH DISEASE (FMD)	(n = 49)	(n = 48)	(n = 26)
Have any of the cattle presented been sick from FMD?			
Yes	60.1% (46.6–72.2%)	76.4% (62.6–86.2%)	7.7% (0.0–18.1%)
Have any of your cattle died from FMD?			
Yes	32.9% (21.5–46.8%)	16.8% (8.8–29.7%)	3.8% (0.0–11.4%)

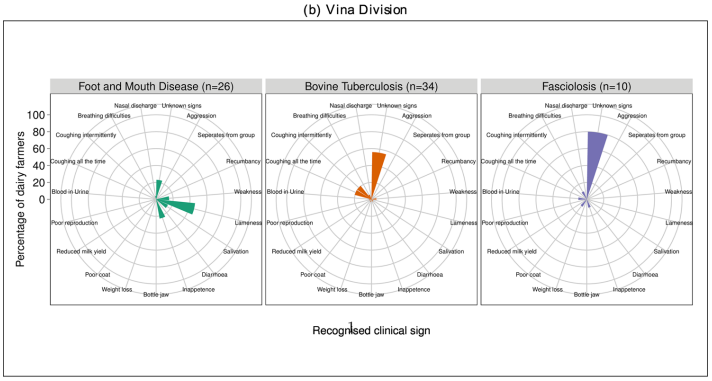
doi:10.1371/journal.pone.0146538.t001



(a) North West Region



(b) Vina Division



(c) Dairy farmers North West Region

Fig 2. Frequency of clinical signs identified for bovine tuberculosis and fasciolosis. Y-axis intervals are for every 20% of cattle keepers "aware" of the disease.

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reported salivation (NWR: 89.8%, CI: 81.2–98.4%, n = 49; VD: 93.8%, CI: 86.8–100%, n = 48)) and lameness (NWR: 81.6%, CI: 70.7–92.6%, n = 49; VD:77.1%, CI: 65.1–89.1%, n = 48) as

clinical signs of FMD along with inappetence, poor coat, reduced milk yield and separation from the group (Fig 2).

Dairy practices and zoonotic tuberculosis

Milk was consumed by the majority of dairy farmers' (87.0%, CI: 77.1–96.8%) and pastoralists' families in both the NWR (87.7%, CI: 77.6–93.6%) and VD (96.0%, CI: 85.4–99.0%). There were differences in processing and production of milk between dairy farmers and pastoralists. Dairy farmers only processed milk by heating whereas pastoralists processed it by souring or heating. All dairy farmers heated milk for their families (CI: 91.2–100%, n = 40). Processing milk for pastoralist's families was more common in the VD (95.8%, CI: 84.8–98.9%, n = 48) than the NWR (74.0%, CI: 61.7–83.4%, n = 44). Furthermore pastoralists in the VD heated (87.5%, CI: 75.5–94.2%) and soured (87.1%, CI: 75.0–93.8%, n = 48) milk more frequently than in the NWR (60.8% (CI: 47.1–73.0%, n = 44) and 55.4% (CI: 43.8–66.5%, n = 44) respectively).

The majority of dairy farmers were producing milk for non-family members (87.0%; CI: 77.1–96.8%) compared to only 42.2% (CI: 29.4–56.1%) of pastoralists in the NWR and 16.5% (CI: 10.0–26.1%) in the VD. However, interestingly only 27.5%, (CI: 13.5–41.5%, n = 40) of dairy farmers processed milk for sale compared to 46.9% (CI: 27.6–76.1%) in the NWR and 9.0%, (CI: 1.3–41.0%, n = 9) in the VD. For those that did process for non-family members, dairy farmers reported heating and in the NWR 41.8% (CI: 23.5–62.7%, n = 21) and VD 9.0% (CI: 1.3–41.0%, n = 9) of pastoralists reported souring milk for non-family members. Dairy farmers and pastoralists produced butter and yoghurt. Fewer dairy farmers (8.7%, CI: 0.5–16.9%) produced butter than pastoralists in the NWR (65.7%, CI: 57.7–76.7%) and VD (75.4%, CI: 62.6–84.9%). Yoghurt was produced by 61.4%, (CI: 50.0–71.7%) of pastoralists in the NWR, 83.6%, (CI: 70.8–91.4%) in the VD and 45.7%, (CI: 31.1–60.2%) of dairy farmers. The median time for milk being soured was one day for pastoralists in the NWR (IQR: 1–2 days) and VD (IQR: 1 day).

Awareness of disease transmission via milk was reported by about a quarter of pastoralists in the NWR (28.3%, CI: 17.3–42.6%) and VD (26.1%, CI: 15.6–40.3%) and about half of the dairy farmers (56.5%, CI: 42.0–71.0%). However, only a small proportion of NWR and VD pastoralists understood that bTB could be transmitted to people through milk, 9.7% (CI: 4.1–21.1%) and 2.0% (CI: 0.3–12.3%) respectively compared with 21.7% (9.7–33.8%) of dairy farmers. Note all descriptive results are also displayed in tabular format (S2 File).

Multivariable regression models for disease awareness

Multivariable models were developed to identify factors associated with the awareness of herdsmen and farmers of the three difference diseases discussed in the questionnaire. The pastoralist and dairy farmers were modelled separately as the husbandry systems were so different. Backward stepwise selection of final models is demonstrated in Table 2. Table 3 shows the final model for bTB awareness and interestingly it suggests that pastoralists in the VD are much less likely to be aware of bTB than those in the NWR. Awareness of fasciolosis and FMD in pastoralists was not associated with Region. For the dairy farmers awareness of bTB, fasciolosis and FMD were all associated with being male (Table 3). There was no association with schooling, ethnic group, age, years kept cattle, job type, milk-processing practices or trading cattle in either pastoralists or small-scale dairy farmers.

Discussion

This study describes the knowledge of bovine tuberculosis, cattle management and milk processing practices amongst pastoralist and small-scale dairy farmers in two Regions of

Table 2. Comparison of mixed-effects logistic regression risk factor models for disease awareness. **(a)** "Are you aware of a disease in cattle called bTB?" (BTBYNU) in pastoralists (n = 100), **(b)** "Are you aware of a disease in cattle called bTB?" (BTBYNU) in dairy farmers (n = 46), **(c)** "Are you aware of a disease in cattle called fasciolosis?" (LFLYNU) in dairy farmers (n = 46,) and **(d)** "Are you aware of a disease in cattle called foot and mouth disease?" (FMDYNU) in dairy farmers (n = 46). Explanatory variables included are OWNSEX (Gender), OWNEDU (Education level), OWNETH (Ethnic group), OWNAGE (Age), OWNCTY (Years worked with cattle), WHOYOU (Job title for pastoralist only), FAMTRT (Do you treat milk?), BUYSAL (Do you trade cattle? For pastoralist only), strata1 (Pastoralist study site) and SUBDIV (Dairy farmer cooperative group). Selected model = *

(a) Pastoralist—Are you aware of a disease in cattle called bTB? **(BTBYNU) (n = 100)**				
Model	**K**	**AIC**	**AICc**	**ΔAIC**
BTBYNU~1 + OWNSEX + OWNEDU + OWNETH + OWNAGE + OWNCTY + WHOYOU + FAMTRT + BUYSAL + strata1	18	148.29	156.84	29.58
BTBYNU~1 + OWNSEX + OWNEDU + OWNETH + OWNCTY + WHOYOU + FAMTRT + BUYSAL + strata1	17	146.43	153.90	26.64
BTBYNU~1 + OWNSEX + OWNEDU + OWNETH + OWNCTY + WHOYOU + BUYSAL + strata1	15	142.67	148.38	21.12
BTBYNU~1 + OWNSEX + OWNEDU + OWNETH + WHOYOU + BUYSAL + strata1	13	139.26	143.49	16.23
BTBYNU~1 + OWNSEX + OWNEDU + OWNETH + WHOYOU + strata1	11	135.79	138.79	11.53
BTBYNU~1 + OWNSEX + OWNETH + WHOYOU + strata1	8	132.43	134.01	6.75
BTBYNU~1 + OWNSEX + OWNETH + strata1	6	130.37	131.27	4.01
BTBYNU~1 + OWNSEX + strata1	3	128.58	128.83	1.57
BTBYNU~1 + OWNSEX	2	138.31	138.43	11.17
BTBYNU~1 + strata1*	2	127.14	12.26	0.00
(b) Dairy farmer—Are you aware of a disease in cattle called bTB? **(BTBYNU) (n = 46)**				
Model	**K**	**AIC**	**AICc**	**ΔAIC**
BTBYNU~1 + OWNSEX + OWNEDU + OWNAGE + OWNCTY + FAMTRT + SUBDIV	11	57.90	65.90	16.29
BTBYNU~1 + OWNSEX + OWNEDU + OWNCTY + FAMTRT + SUBDIV	9	55.46	60.46	10.85
BTBYNU~1 + OWNSEX + OWNCTY + FAMTRT + SUBDIV	6	52.64	54.79	5.18
BTBYNU~1 + OWNSEX + OWNCTY + FAMTRT	4	50.17	51.15	2.54
BTBYNU~1 + OWNSEX + FAMTRT*	3	49.04	49.61	0.00
BTBYNU~1 + FAMTRT	2	54.97	55.25	5.64
BTBYNU~1 + OWNSEX	2	50.12	50.40	0.79
(c) Dairy farmer—Are you aware of a disease in cattle called fasciolosis? **(LFLYNU) (n = 46)**				
Model	**K**	**AIC**	**AICc**	**ΔAIC**
LFLYNU~1 + OWNSEX + OWNEDU + OWNAGE + SUBDIV + FAMTRT + OWNCTY	11	44.44	52.44	12.63
LFLYNU~1 + OWNSEX + OWNEDU + SUBDIV + FAMTRT + OWNCTY	9	41.77	46.77	6.96
LFLYNU~1 + OWNSEX + OWNEDU + SUBDIV + OWNCTY	8	39.95	43.84	4.03
LFLYNU~1 + OWNSEX + SUBDIV + OWNCTY	5	38.31	41.90	2.08
LFLYNU~1 + OWNSEX + OWNCTY*	3	37.75	39.81	0.00
LFLYNU~1 + OWNSEX	2	42.79	40.76	0.95
LFLYNU~1 + OWNCTY	2	45.48	50.44	10.53
(d) Dairy farmer—Are you aware of a disease in cattle called foot and mouth disease? **(FMDYNU) (n = 46)**				
Model	**K**	**AIC**	**AICc**	**ΔAIC**
FMDYNU~1 + OWNSEX + OWNEDU + OWNAGE + OWNCTY + FAMTRT + SUBDIV	11	69.13	77.13	17.15
FMDYNU~1 + OWNSEX + OWNAGE + OWNCTY + FAMTRT + SUBDIV	8	64.22	68.73	8.75
FMDYNU~1 + OWNSEX + OWNAGE + OWNCTY + FAMTRT	6	61.69	63.89	3.91
FMDYNU~1 + OWNSEX + OWNCTY + FAMTRT	4	61.35	62.32	2.34
FMDYNU~1 + OWNSEX + OWNCTY*	3	59.41	59.98	0.00
FMDYNU~1 + OWNSEX	2	61.31	60.47	0.49
FMDYNU~1 + OWNCTY	2	60.19	61.59	2.11

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Table 3. Final mixed-effects logistic regression risk factor models for disease awareness. **(a)** "Are you aware of a disease in cattle called bTB?" (BTBYNU) in pastoralists (n = 100), **(b)** "Are you aware of a disease in cattle called bTB?" (BTBYNU) in dairy farmers (n = 46), **(c)** "Are you aware of a disease in cattle called fasciolosis?" (LFLYNU) in dairy farmers (n = 46,) and **(d)** "Are you aware of a disease in cattle called foot and mouth disease?" (FMDYNU) in dairy farmers (n = 46). Explanatory variables included are OWNSEX (Gender) and strata1 (Pastoralist study site).

(a) Pastoralist—Are you aware of a disease in cattle called bTB? **(BTBYNU) (n = 100)**				
Final model: BTBYNU~1 + strata1				
Variables	**Levels**	**Odds ratio**	**95% CI**	**p value**
strata1	North West Region	1		
	Vina Division	0.23	0.09–0.53	<0.01
(b) Dairy farmer—Are you aware of a disease in cattle called bTB? **(BTBYNU) (n = 46)**				
Final model: BTBYNU~1 + OWNSEX				
Variables	**Levels**	**Odds ratio**	**95% CI**	**p value**
OWNSEX	Female	1		
	Male	8.63	1.86–63.21	0.01
FAMTRT	No	1		
	Yes	6.46	0.81–68.16	0.09
(c) Dairy farmer—Are you aware of a disease in cattle called fasciolosis? **(LFLYNU) (n = 46)**				
Final model: LFLYNU~1 + OWNSEX				
Variables	**Levels**	**Odds ratio**	**95% CI**	**p value**
OWNSEX	Female	1		
	Male	21.91	3.05–461.65	<0.01
OWNCTY	> = 5 years	1		
	<5 years	0.12	0.01–0.65	0.04
(d) Dairy farmer—Are you aware of a disease in cattle called foot and mouth disease? **(FMDYNU) (n = 46)**				
Final model: FMDYNU~1 + OWNSEX				
Variables	**Levels**	**Odds ratio**	**95% CI**	**p value**
OWNSEX	Female	1		
	Male	3.74	1.01–14.77	0.05
OWNCTY	> = 5 years	1		
	<5 years	3.27	0.81–14.75	0.10

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Cameroon. Such studies are important because as WHO moves towards its aim of eradicating tuberculosis by 2050 [52]; the relative importance of zoonotic tuberculosis (zTB) caused by *M. bovis* may increase particularly in sub-Saharan Africa with its developing dairy industries [53]. In this Region future interventions preventing the zoonotic spread of tuberculosis are likely to be aimed at preventing transmission in milk and reducing the herd prevalence. Milk producers and cattle herds will be the targets of control strategies. Hence improving our understanding of livestock keepers knowledge of bTB, cattle management and milk processing practices which may favour *M. bovis* transmission are important.

In common with many countries in sub-Saharan Africa, Cameroon has a small-scale dairy industry based on imported *Bos taurus* and their cross-breeds [32]. This study indicates that the herd structure, contact and movement patterns of these herds and the awareness of bTB amongst dairy farmers were very different from the traditional *Bos indicus* keeping pastoralists. Small-scale dairy farmers had small numbers of Holstein-Friesian cows with little reported contact with other herds or wildlife. Although the control of tuberculosis in this population may appear tractable, e.g. by closing herds, using artificial insemination and test and slaughter, there are a number of caveats to this. These herds were set up with a limited number of imported animals from Ireland and Kenya. Both of these countries report endemic bTB and in the absence of a perfect test, importation of bTB as well as cattle cannot be discounted. The

occurrence of pseudo-vertical transmission means that one infected herd may have given rise to a number of secondarily infected herds [16]. For example as there is a reliance on using shared bulls as the majority of dairy herds use natural service and an infected bull could potentially infect numerous herds. Furthermore although dairy herd sizes are small; test and slaughter policies would have a major impact on the livelihoods of these farmers and sustainable public health measures should be considered in this context.

In comparison the control of tuberculosis amongst the large pastoralist herds, by movement and contact restrictions, presents a much greater challenge and is probably impossible in the absence of an effective vaccine. These herds are relatively large and had daily contact with other herds and wildlife especially antelopes and warthogs. Additionally the practice of transhumance dramatically increased the number and range of contacts with other herds and wildlife. The importance of wildlife contact is indicated by studies in Zambia where the prevalence of bTB in cattle was proportional to its prevalence in wildlife [54]. Wildlife in South Africa have been shown to carry the same spoligotypes of *M. bovis* as cattle but transmission ecology is unclear[55]. Species of antelope in Cameroon include eland (*Taurotragus spp*), roan antelope (*Hippotragus spp*), korrigum (*Damaliscus spp*), kob (*Kobus spp*) and duiker (*Cephalophus spp)* [56,57]. These species of antelope are different to those in South Africa and interaction between cattle and wildlife is poorly defined in Cameroon. As wildlife could be a reservoir host of bTB; susceptibility of Cameroonian antelope and other wildlife species, such as buffalo and warthogs, to *M. bovis* requires further investigation [58].

These observations on cattle husbandry assist in identifying priorities for bTB research in this environment. Biologically, there is a need to understand the relative importance of the different routes of transmission and the susceptibility and infection status of potential reservoir hosts. Socio-economically there is a need to understand the drivers for transhumance as there appeared to be geographical and ethnic differences in the practice between communities. Highlighting a potential problem if disease free zones were to be established where transhumance is undertaken. The reasons for this difference in grazing practices is unclear. It may reflect pressure on land or different cultural traditions. Conflict between pastoralists and arable farmers in the NWR has been recognised as a perennial problem and the subject of a number of studies of the competition for the natural resources of land and water [59–62]. Cultural difference in the nomadic activities of the Mbororo and sedentary Fulbe are also well recognised [29]. But there is evidence that the frequency of transhumance is declining. In a previous study of pastoralists in the VD carried out in 2000, 29.2% reported transhumance compared with 6% in the current study which would be of benefit for bTB control [63].

In addition to understanding cattle demography and contact networks, the awareness and knowledge of pastoralists and small-scale dairy farmers about bTB will be an important component of any bTB control scheme. Awareness of bTB amongst pastoralists was associated with Region with a greater portion being aware in the NWR than VD. This regional difference was surprising. It may be because pastoralists had worked with cattle for longer in the NWR than the VD. However this seems unlikely because all pastoralists were aware of FMD and fasciolosis, endemic diseases in both sites, and there was no difference in awareness between groups. It is possible that they encountered bTB more frequently in the NWR either from clinical cases or abattoir condemnations. A previous abattoir study reports a higher bTB prevalence in the NWR than the VD potentially supporting this theory[25]. Also a similar proportion of pastoralists, who were aware of bTB, in each area were able to identify clinical signs consistent with bTB; such as coughing, weight loss, poor coat and weakness. It is important to note that, unlike FMD where the clinical signs are relatively pathognomonic, that clinical signs of bTB may not develop in an infected animal despite the presence of severe pathology [2,64,65]. Yet detections of TB lesions in abattoirs may raise awareness as pastoralists are notified of carcase

condemnations at abattoirs, as the vending herdsman and purchasing butcher share the financial loss of the condemnation. Furthermore veterinary staff, inspecting slaughtered cattle, may inform herdsmen about clinical signs of bTB and subsequently increase awareness.

Awareness of bTB amongst dairy farmers was associated with gender, with male dairy farmers 6 times more likely to be aware of bTB than females. Interestingly this pattern was repeated for the other diseases, with male dairy farmers also more aware of fasciolosis and FMD. Potentially women did not benefit from the cattle husbandry training given when dairy cooperatives and the reasons for this are unclear. In other low income countries, were there is poor knowledge of livestock diseases, it has been shown that education programs have not targeted the primary individuals involved in livestock rearing [66]. Other studies have shown that livestock education programs are often directed at men as they are presumed to be the individual primarily involved in livestock production [67]. Yet this is not the case across all livestock rearing communities and this is demonstrated in Cameroon with half of dairy farmers being female. Taking into account the strong movement to encourage women into livestock keeping, as a method of poverty alleviation, it is important that any future bTB educational programs are not gender biased [68]. Interestingly dairy farmers appeared to be less able to identify clinical signs of bTB than pastoralists. This was also a consistent trend for fasciolosis and to a lesser extent FMD. It is unclear why such a high percentage of farmers who had heard of these diseases were unaware of their clinical signs. It may reflect the frequency of exposure of dairy farmers to these diseases with dairy cattle being managed as individuals with little mixing with other cattle. Reasons for inconsistencies in bTB awareness are unclear and further research is required in this area and may hinder the acceptance of future control programs in certain communities.

One of the main reasons for controlling bTB is due to its potential of being a milk-borne zoonosis [6,13]. Unsurprisingly, with regular access to milk, there appears to be widespread milk consumption and processing of milk in pastoralist and dairy farmer's families. About half of the dairy farmers were aware of milk borne disease but interestingly few were aware of milk borne transmission of *M. bovis*. The proportion of pastoralists who were aware of zoonotic TB transmission in milk was even lower than dairy farmers. This suggests that improving knowledge and awareness of milk borne transmission is an important message in any public health program to control zoonotic TB. Transmission of zoonotic TB, through consumption of infected milk, can be controlled by heating milk for example through pasteurisation [69]. Although the prevalence of heating milk was collected in this study we have no information on the temperature to which it was heated e.g. it is possible that it was always heated to a sufficient temperature and time to destroy *M. bovis*. Data on the duration of heating were collected but in the absence of information on the volume and temperature of milk being heated these are difficult to interpret. However there were some interesting differences in the patterns of heating. All dairy farmers heated milk for their families but just over a quarter heated milk prior to sale. As most dairy farmers were producing milk for non-family members this represents an important potential route of transmission of bTB. It is unclear why this difference should exist in milk consumed by the family and by non-family members. It may reflect the financial cost of purchasing fuel to heat the milk or a preference by consumers for raw milk e.g. because of ease in detecting its freshness or because they wish to preserve milk by converting it into yogurt or cheese. Pastoralists also heated milk for family consumption. In contrast to dairy farmers, fewer pastoralists produced milk for non-family members especially in the VD. This difference may reflect the recognition by pastoralists in the NWR of the financial value of the developing dairy market in the Region. A similar percentage of NWR pastoralist and dairy farmers used artificial insemination, with Holstein-Friesian semen, compared to VD pastoralists. Suggesting an overall drive for increased milk production by both cattle rearing groups in the NWR. In

addition to heating, souring milk was common amongst pastoralists, especially in the VD, appearing to be not culturally important to dairy farmers preserving milk. There are limited and mixed reports of the efficacy of traditional milk souring techniques to destroy *M. bovis* [70–72]. A study in Zambia showed milk spiked with *M. bovis* and then naturally soured over 1–3 days did not consistently destroy the bacterium [73]. However, a recent study from South Africa suggests that souring is effective if the product is maintained at an adequate temperature and is time dependent [74]. Additionally efficacy of souring may dependent upon pH reached and the bacterial populations [75,76]. Hence the final soured product varies with different souring techniques and souring methods vary across sub-Saharan Africa, In this study pastoralists soured milk for a median of one day. Souring for one to two days, without a starter culture, has also been previously reported in Cameroon but it is unclear if the same techniques are used homogenously across the country [32,75]. Unlike heating, souring requires no external energy source or additional financial investment so maybe useful in low-income settings if done correctly [74]. Hence identifying the variety of souring techniques, their efficacy and awareness could inform future public health education programs.

In conclusion, this study has described the cattle husbandry, dairy practices and knowledge of bTB in pastoralist and small-scale dairy farming communities. The presence of different cattle rearing systems within a country, pose different challenges to be taken into account when developing bTB control programs. The study has also identified a need for investigation of current milk processing practices to determine whether they are effective in inactivating *M. bovis* in countries where milk processing is unregulated. Overall the gap in bTB knowledge and awareness identified may hinder future *M. bovis* control in cattle and people. Looking to the future targeted TB education programs within cattle rearing communities could be potentially beneficial to raise the awareness of zTB and improve peoples understanding of mitigating actions such as boiling and souring milk.

Supporting Information

S1 File. FieldQuestionnaire_PLOSone.pdf. Questionnaire, in Fulfulde language, used in the pastoral and dairy cross-sectional studies.
(PDF)

S2 File. DescriptiveStatistics_PLOSone.docx. Reference table of descriptive data analysis from pastoral and dairy cross-sectional studies.
(DOCX)

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Author Contributions

Conceived and designed the experiments: MB RK LN VT KM IH WA SH MS EN VN. Performed the experiments: RK SH EN. Analyzed the data: RK MB IH SM. Contributed reagents/materials/analysis tools: RK MB IH SM MS. Wrote the paper: RK KM MB AM VT IH SM SH EN MS VN LN WA.

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Chapter 8

Comparing the interferon-gamma assay to the SCITT to describe the epidemiology of bovine tuberculosis in Cameroon.

8.1 Introduction

In order to understand the potential impact of bTB within Cameroon it is vital to describe its epidemiology, not only to describe the prevalence within the cattle population but also to highlight potential risk factors to facilitate bTB control. Previous estimates of bTB prevalence, using PME and ante-mortem diagnostics, vary between 0.1-40% (120; 77; 366; 114; 75; 115; 116; 118; 117). Abattoir surveys may be useful to highlight the presence of bTB within a population and assess diagnostic test performance however the sample may not be representative of the cattle population as a whole (Appendix H). Furthermore, PME is insensitive and may lead to an underestimation of the prevalence, particularly missing early stage infections (48). In a limited-resource setting like Cameroon, detection of clinical signs of bTB in live cattle could highlight presence of bovine disease in cattle populations. However, the association between recognised bTB clinical signs and bTB positive cattle has been minimally investigated.

The variation between ante-mortem diagnostic prevalence estimates of bTB in Cameroon, is partly related to test detecting different aspects of the immune response to *M. bovis* and performance of the diagnostics in this setting (Chapter 4). Bovine *M. bovis* infections predominately stimulate CMI responses(256; 140; 264) and ante-mortem diagnostic tests used to detect part of this CMI response include the in-vitro IFN-γ assay and the in-vivo tuberculin skin tests (SIT and SCITT)(376; 193; 48). The SCITT is recognised by the OIE as the primary diagnostic test for bTB diagnosis (45), with a high specificity (median: 99.5%; CI: 78.8-100%) (78; 371). However, the SCITT has a relatively low sensitivity (median: 83.9%; CI: 52-100%) resulting in the risk of false negative animals and could result underestimation of the bTB prevalence. The SCITT and IFN-γ assay measure

different aspects of the CMI response(368; 181) and subsequently do not detect the same population of bTB positive cattle. The IFN-γ assay detects the predominant Th1 immune response to *M. bovis* from 2 weeks post infection (260), with a higher sensitivity (Median: 87.6%; CI: 73.0-100%) than the SCITT and comparable specificity (Median: 96.6%; CI: 85.0-99.6%). Hence the assay may provide a more accurate estimate of the bTB prevalence in Cameroon. "Parallel" testing is the combination of being positive on one or both tests and the SCITT and IFN-γ assay have been used in "parallel" to improve overall sensitivity in some settings (167; 46; 432). Using the tests in combination has not been explored in Cameroon and may improve the accuracy of prevalence estimates.

Additionally, understanding why these two tests disagree may be useful when interpreting bTB prevalence estimates using only on or the other. In Cameroon *F. gigantica* co-infections have been shown to reduce the sensitivity of the IFN-γ assay by $\sim$20% (Chapter 5). Therefore the *F. gigantica* status of cattle, should be taken into account when interpreting the IFN-γ assay, to avoid underestimating bTB prevalence.

Risk factors for bTB positivity may vary within pastoral and dairy cattle populations in Cameroon due to differences in management practices (Chapter 7). Identifying these differences is likely to be important to highlight potential future bTB control options within these production systems and risk to public health within cattle rearing communities in Cameroon. Considering IFN-γ positive cattle are likely to go on to shed *M. bovis* (433) using the IFN-γ assay to estimate prevalence and identify risk factors for bTB positive cattle could be useful in identifying routes of *M. bovis* introduction into herds.

This chapter compares the prevalence estimates of bTB using the IFN-γ assay, the SCITT and the "parallel" test combination for pastoral (NWR and VD) and dairy

(NWR only) cattle populations. The level of agreement between the IFN-γ assay and the SCITT is investigated along with the factors that lead to their diagnostic disagreement. The potential impact of the *F. gigantica* co-infection of reducing IFN-γ assay diagnostic test sensitivity on prevalence estimates is explored. Additionally the association of presence of clinical signs and being bTB positive is explored. Finally potential risk factors for pastoral and dairy cattle being bTB positive, using the IFN-γ assay, are investigated in an attempt to further understand bTB epidemiology in Cameroon.

8.2 Materials and methods

8.2.1 Cross-sectional studies

Data collected in the pastoral and dairy farmer cross-sectional studies were used for the analysis in this chapter. Study designs and sampling methodology were described in detail in section 3.2.2. In total 100 pastoral (50 NWR and 50 VD; 14-15 cattle sampled per herd) and 46 dairy herds (All NWR; 1-4 cattle per herd) were sampled. A combination of bTB diagnostic tests were performed in the pastoral and dairy cross-sectional studies. All sampled cattle have IFN-γ assay results (NWR n=750, VD n=748, Dairy n=60). All pastoral cattle in the NWR (n=750) and dairy cattle (n=60) have a SCITT result. Seven cattle in the VD are missing a SCITT result as they could not be found, after a lightening storm, when the SCITT reaction was to be interpreted (n=741). Diagnostic test methodology and interpretation formulae are described in section 3.3.

Pastoral and dairy cattle data are used to describe the cattle sampled, estimate the bTB prevalence and risk factors for bTB positivity. Analysis of agreement and disagreement, between the IFN-γ assay and SCITT, was based on the pastoral cross-sectional only due to differences in management of dairy cattle precluding their inclusion.

8.2.2 Statistical analysis

Descriptive statistics

Statistical analyses were performed using packages and functions in R (343). Graphics were produced using the *ggplot2* package (344). Maps were drawn using

QGIS 2.2® (345) and shape files were obtained from the open access GADM database of Global Administrative Areas (www.gadm.org). Proportions and prevalences were calculated with 95% CIs to compare differences between pastoral, NWR and VD, and dairy cattle samples (348). The packages *svymean*, *confint* and *svyby* functions were used to calculate prevalences and to account for the design effects (346). For dairy cattle bTB prevalence was estimated in a similar manner accounting for the simple study design.

Diagnostic agreement was investigated for the IFN-γ assay and SCITT for pastoral cattle only stratified by study site (NWR n=750; VD n=748). Firstly agreement of raw diagnostic test results was investigated using scatter plots comparing two tests at a time (section 3.3). Percentage agreement and Cohens kappa statistics were used to quantify agreement between the two tests and the criteria for interpretation is described in section 3.5.2. Functions used to calculated these values were the *agree*, *kappa2* and *rater.bias* in the *irr* package (359).

Factors associated with disagreement between the IFN-γ assay and SCITT for pastoral cattle were stratified by study site (NWR n=750; VD n=748). Multivariable logistic regression (MLR) models to investigate the two forms of IFN-γ assay and SCITT binary disagreement:

1. **IFN-γ assay positive** and SCITT negative (Figure 8.1).
2. **IFN-γ assay false negative** and SCITT positive (Figure 8.2).

For each study site two subsets of each result of the two diagnostic tests were used to produce four disagreement models. The conservative approach to model selection is outlined previously (Section 3.5.3). The outcome variable was the binary result for the alternative test with the result of interest being the contrary binary result. e.g. subset positive and outcome variable negative. Only intrinsic animal level variables

were included as explanatory variables in model selection. The DS variable was recategorised to <3 years (DS<2) and ≥3 years (DS≥2). The breed variable was recategorised to Gudali (Gudali breed), Fulani (Red Fulani or white Fulani), mixed breed and Holstein-Friesian cattle. Models were constructed using the *glmer* function in the *lme4* package (434). In the final models herd (HER_ID) was always included in the model as a random effect to take into account for clustering within the herd. All relevant explanatory variables that are considered to be potentially biologically significant were included in models and interactions investigated (360). Model selection was based on the Akaike Information Criterion (AIC) and the best model was selected using the lowest AIC. Final model selection was verified by computing ΔAIC using the package *AICcmodavg* and *modavg* function (363). The p value, odds ratio with 95% CI for explanatory variables are also calculated.

Figure 8.1: **Diagrammatic representation of the subsetting of the data to investigate test disagreement between positive IFN-gamma assay ($\geq$0.1) and negative SCITT ($\leq$2mm) for the North West Region and Vina Division.**
Model a) Subset of all cattle with a positive IFN-γ assay response highlighted in orange with SCITT as the dependent variable. Model b) Subset of all cattle with a negative SCITT response highlighted in purple with IFN-γ assay as the dependent variable. Black areas indicates "positive" (Disparate results) and white areas indicates "negative" (Agreeing results) of the dependent variable.

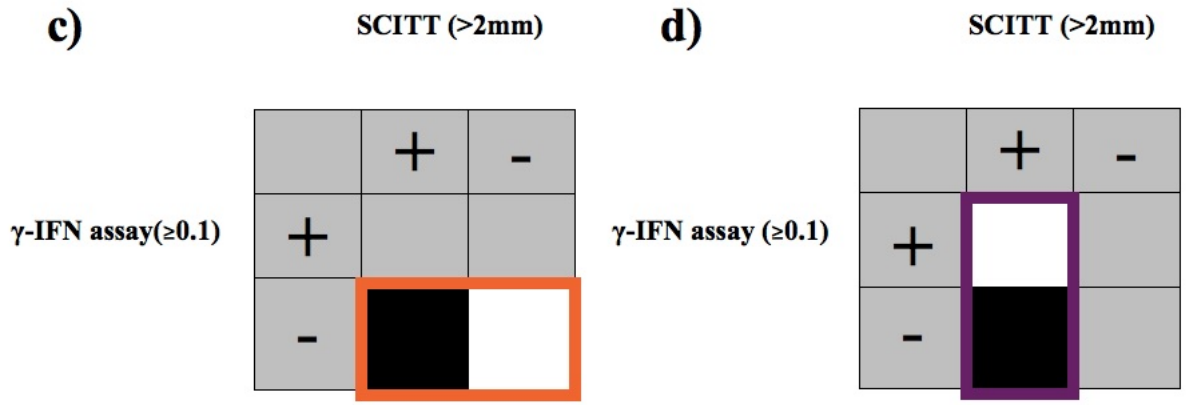


Figure 8.2: **Diagrammatic representation of the subsetting of the data to investigate test disagreement between negative IFN-gamma assay (<0.1) and positive SCITT (>2mm) for the North West Region and Vina Division.**
Model c) Subset of all cattle with a negative IFN-γ assay response highlighted in orange with SCITT as the dependent variable. Model d) Subset of all cattle with a positive SCITT response highlighted in purple with IFN-γ assay as the dependent variable. Black areas indicates "positive" (Disparate results) and white areas indicates "negative" (Agreeing results) of the dependent variable.

Descriptive statistics were calculated to investigate the patterns of clinical signs associated with bTB test positivity for pastoral cattle only (NWR n=750; VD n=748). Correlations between pairs of clinical signs were explored using Spearmans correlation coefficient (r) calculated using the *corrplot* function of the *corrplot* package. Odds ratios and respective confidence intervals were calculated to determine if clinical signs were associated with bTB positive animals, either by the IFN-γ assay or SCITT, using the *oddsratio* function in the *epitools* package (435).

The impact of *F. gigantica* exposure on the estimated bTB prevalence was explored in pastoral and dairy cattle. Bovine tuberculosis status was determined using the IFN-γ assay and exposure to *F. gigantica* was determined using the *F. gigantica* antibody ELISA (Chapter 6). Firstly the prevalence of *F. gigantica* was compared to the bTB prevalence in the NWR Divisions and VD sub-divisions to assess for an association. Secondly the true bTB prevalence in the NWR and VD was estimated to account for IFN-γ assay specificity (From the published median of 96.6% (78)) and sensitivity in two situations:

1. Where *F. gigantica* had no effect with a test sensitivity of 51.7% (Estimated previously in Chapter 5).
2. Assuming 100% exposure to *F. gigantica* had an reduced test sensitivity by 20.3% to 31.4% (Chapter 5).

True prevalence (134) was estimated using the *epi.prev* function in the *epiR* package (351).

Secondly to identify risk factors for being IFN-γ positive in pastoral (NWR and VD; n=1498) and dairy cattle (n=60) two MLR models were constructed using the *glm* function in the *stats* package (343). A backwards stepwise model selection approach was conducted as described previously when investigating $\geq$10 possible explanatory

variables (Section 3.5.3 and (361)). The main outcome variable was the binary variable IFN-γ assay result. Explanatory variables were screened from data collected at individual (Appendix F) and herd level (Appendix D). Animal level variables were re-categorised as previously described in this section.The steps taken in final model selection, for the pastoral and dairy cattle models, was conducted as follows:

1. Univariate logistic regression was used to screen explanatory variables. Variables were screened that were deemed biologically plausible in bTB transmission. Correlation between variables was undertaken by calculating the phi coefficient using the *psych* package (364). If phi was $\geq$0.5 two variables were considered correlated and the value with the highest p value was selected. Variables were included in the final MRL selection if their p value $\leq$0.2 (Appendix J).

2. A backwards stepwise approach was used to find the best fitting model to describe the dataset when constructing the model with assessment for interactions (360). Model selection was based on the AIC and the best model was selected using the lowest AIC. Once the final model was selected each variable from the final model was removed singularly to assess changes in AIC. Final model selection was verified by computing AIC, due to small sample sizes used for models (362), and ΔAIC using the *AICcmodavg* package and *modavg* function (363). In the final model herd (HER_ID) was always included in the model as a random effect to take into account for clustering within the herd. Also to account for clustering by study site, the variable (strata1) was always included in the model as a fixed effect. The p value, odds ratio with 95% CI for explanatory variables were also estimated.

3. The final model was then compared with and without *F. gigantica* serology variable. The variable was considered to have an association if it altered the

significance of the p value of any of the variables by 10% (361).

4. The final MLR model prediction was assessed using ROC analysis (354) and histograms of model residuals were plotted to assess for normality (360).

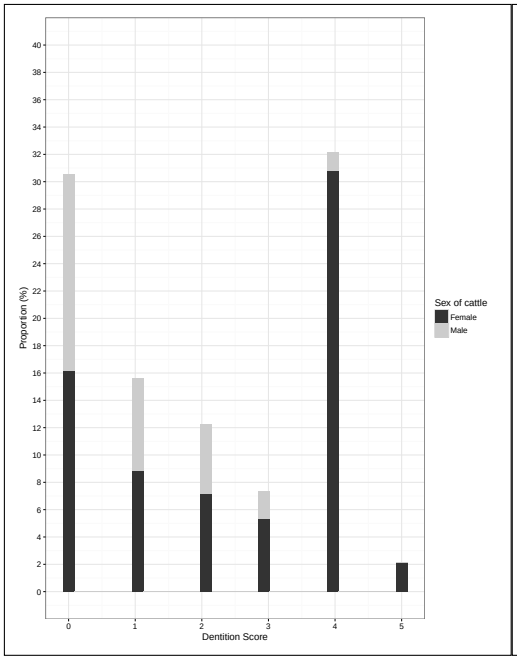
5. For dairy cattle the same method of model selection was used (Appendix J except *F. gigantica* serology was not added due to the low exposure (Chapter 6 and section 8.3).

8.3 Results

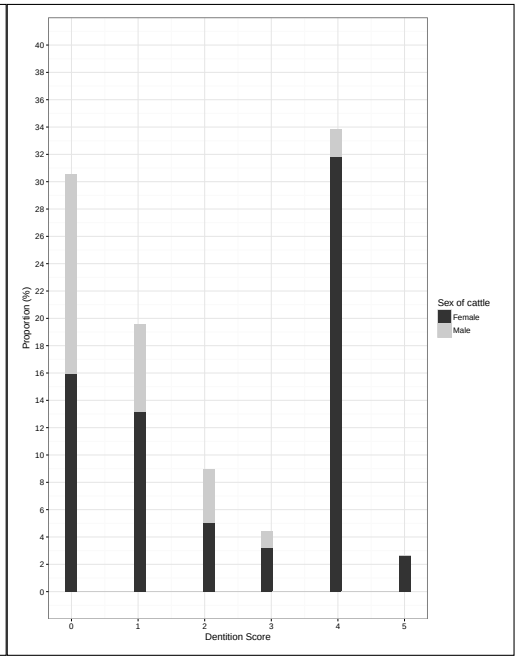
8.3.1 Cattle samples

In total 750 cattle were sampled from 50 herds (15 per herd) in the NWR and 748 cattle from 50 herds (14-15 per herd) in the VD in the pastoral cattle study. In the dairy cross-sectional study 60 cattle (1-4 per herd) were sampled from 46 dairy farmers. The majority of all pastoral and dairy cattle were female (Figure 8.4). In the pastoral cross-sectional study the ratio of male to female cattle was relatively equal in young cattle (<1 DS) and the majority of older cattle ($\geq$4 DS) were female. A majority of dairy cattle were $\geq$2 DS (Table 8.1 and figure 8.4). In general pastoral cattle in the NWR had lower BCSs than pastoral cattle in the VD and dairy cattle. The majority of the dairy cattle had been treated with an anthelmintic in the previous 12 months. Fewer pastoral cattle were treated with an anthelmintic in the previous 12 months and the majority of those treated were less than two years of age (<1 DS) (Table 8.1).

(a) **North West Region** (Pastoral n=750)



(b) **Vina Division** (Pastoral n=748)



(c) **North West Region** (Dairy n=60)

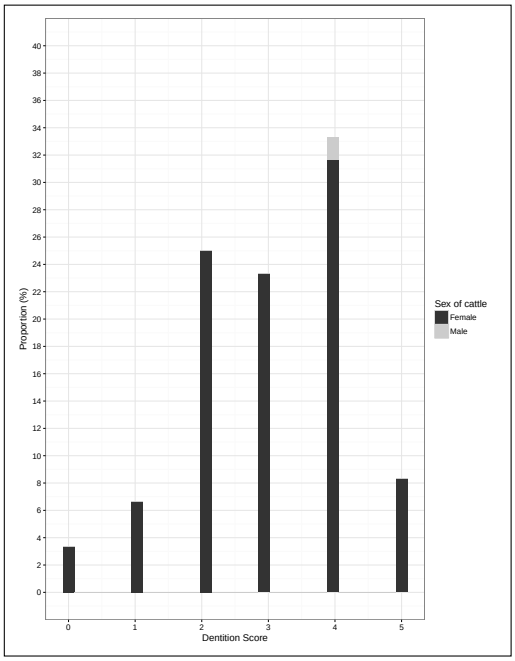


Figure 8.4: **Proportion of sex and dentition score of sampled cattle by study site grouping.**

Proportions by study site are the proportions of total number of cattle per abattoir with a full set of data (North West Region pastoral cattle n=750; Vina Division pastoral cattle n=748; North West Region dairy cattle n=60). Dentition score is re-catagoried for the remainder of this thesis to age <3 years (0-2) and $\geq$3 years (3-5).

	North West Region n=750 (95% CI)	Vina Division n=748 (95% CI)	Dairy n=60 (95% CI)
Sex			
Male	29.7% (26.8-32.7%)	26.5% (22.7-30.8%)	1.7% (0.0-4.9%)
Female	70.3% (67.3-73.2%)	73.5% (69.2-77.3%)	98.3% (95.1-100%)
Age (By dentition score (DS))			
<3 years	59.2% (56.8-61.7%)	59.9% (57.4-62.4%)	35.0% (22.8-47.2%)
≥3 years	40.8% (38.3-43.2%)	40.0% (37.6-42.4%)	65.0% (52.8-77.2%)
Breed			
Holstein Friesian	0.0% (0.0-0.5%)	0.0% (0.0-0.5%)	98.3% (95.1-100%)
Gudali	0.0% (0.0-0.5%)	83.5% (78.1-88.8%)	0.0% (0.0-6.0%)
Mixed Breed	63.9% (54.6-72.2%)	14.6% (10.0-20.7%)	0.0% (0.0-6.0%)
Fulani	36.1% (27.8-45.4%)	2.0% (0.9-4.1%)	0.0% (0.0-6.0%)
Body condition score (BCS)			
Thin (1-2)	58.1% (53.8-62.3%)	24.2% (20.8-28.0%)	25.0% (14.0-36.0%)
Moderate (3)	37.0% (33.4-40.8%)	56.4% (53.0-59.8%)	50.0% (37.2-62.8%)
Fat (4-5)	4.9% (3.1-7.5%)	19.3% (15.9-23.1%)	25.0% (14.0-36.0%)
Anthelmintic treatment in the past 12 months			
Treated	47.3% (39.2-55.4%)	30.9% (25.3-37.1%)	100.0% (94.0-100%)

Table 8.1: **Descriptive summary of pastoral and dairy cattle.**

8.3.2 Agreement between the IFN-gamma assay and SCITT

Considering the pastoral cattle results only, the agreement between the IFN-γ assay ($\geq$0.1 positive cut-off value used from analysis conducted in chapter 4) and the SCITT are compared at >2mm and >4mm cut-off values (used in bTB control programs internationally(45)) by study site (Figure 8.4). Results were plotted for the two study sites with suggested cut-offs values for the SCITT and IFN-γ assay (Figure 8.5). For both study sites agreement between IFN-γ assay and SCITT for a negative results (area shaded yellow) appeared to be consistent. The main disagreement between the two tests was where cattle had positive IFN-γ assay and negative SCITT at >2mm (NWR= 7.3% CI: 5.6-9.4%; VD= 4.9% CI: 3.4-6.7%) or >4mm (NWR= 9.2% CI: 7.2-11.5%; VD= 5.1% CI: 3.7-7.0%) results. Few cattle were IFN-γ assay negative and SCITT positive at >2mm (NWR= 1.9% CI: 1.0-3.1%; VD= 1.5% CI: 0.7-2.6%) and >4mm (NWR= 0.3% CI: 0.1-1.0% ; VD= 0.4% CI: 0.1-1.2%) positive cut-off values. Overall percentage agreement was above 90.0%, for both study sites and both SCITT positive cut-off values (Table 8.2). For the NWR there was no difference between Cohens kappa statistic between IFN-γ assay and the SCITT at >2mm and >4mm positive cut-off values. Although agreement from the Cohens kappa statistic, between the SCITT (>2mm) and the IFN-γ assay implied a "fair-moderate" agreement (κ.21-0.4= fair agreement; κ.41-0.6= moderate agreement). For the VD there was also no difference between Cohens kappa statistic for the IFN-γ assay and SCITT with "poor-moderate" agreement reported for either >2mm and >4mm cut-off values (κ.01-0.2= poor agreement; κ.21-0.4= fair agreement; κ.41-0.6= moderate agreement). As the Cohens kappa statistic did not differ between the study sites, and previous studies highlighting the use of >2mm positive cut-off (115), the >2mm positive cut-off value was chosen to be used for the remainder of the analysis.

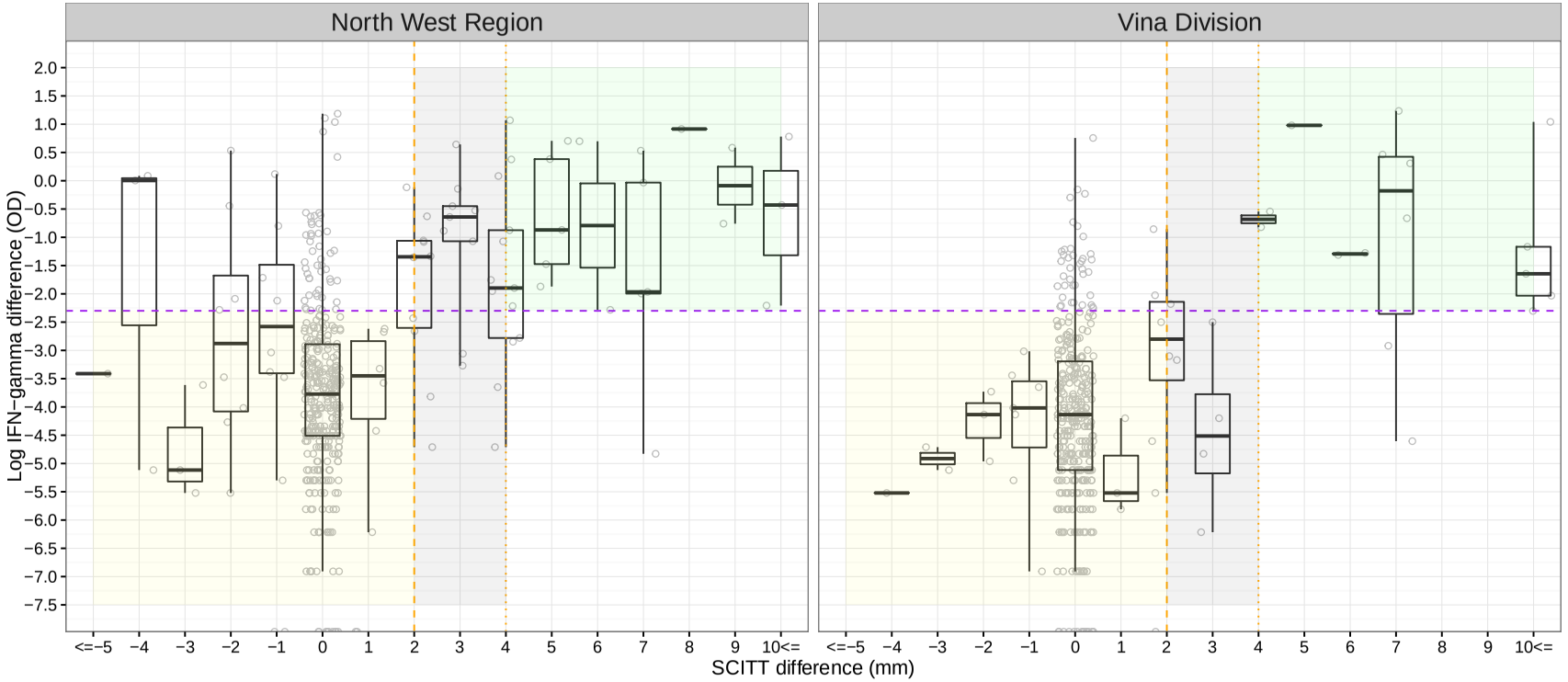


Figure 8.5: **Boxplot of the IFN-gamma assay and SCITT raw difference between avian and bovine reactions in pastoral cattle in the North West Region (n=750) and the Vina Division (n=741).**

For the IFN-γ assay results are displayed on a log scale for clarity and $\geq$0.1 positive cut-off value are shown (Horizontal purple dashed line; ln(0.1) = -2.3). For the SCITT >2mm (Horizontal orange dashed line) and >4mm (Horizontal orange dotted line) positive cut-off values are shown. The green area denotes the test positive cattle for IFN-γ assay ($\geq$0.1) and SCITT (>4mm). The grey area denotes proportion of additional test positive cattle for IFN-γ assay ($\geq$0.1) and SCITT ($\geq$2mm). The yellow area denotes test negative cattle for IFN-γ assay (<0.1) and SCITT ($\leq$2mm).

North West Region					
IFN-gamma assay	**SCITT (>2mm) (n=750)**		**Percentage agreement**	**Cohens kappa statistic**	**Cohens kappa statistic 95% CI**
		+	-		
>=0.1	+	30	55	90.8%	0.42	0.31-0.53
	-	14	651		
IFN-gamma assay	**SCITT (>4mm) (n=750)**		**Percentage agreement**	**Cohens kappa statistic**	**Cohens kappa statistic 95% CI**
		+	-		
>=0.1	+	16	69	90.5%	0.28	0.17-0.39
	-	2	663		
Vina Division					
IFN-gamma assay	**SCITT (>2mm) (n=741)**		**Percentage agreement**	**Cohens kappa statistic**	**Cohens kappa statistic 95% CI**
		+	-		
>=0.1	+	13	36	93.7%	0.33	0.18-0.47
	-	11	681		
IFN-gamma assay	**SCITT (>4mm) (n=741)**		**Percentage agreement**	**Cohens kappa statistic**	**Cohens kappa statistic 95% CI**
		+	-		
>=0.1	+	11	38	94.5%	0.33	0.18-0.47
	-	3	689		

Table 8.2: **Comparisons of agreement and Cohens kappa statistic between IFN-gamma assay ($\geq$0.1) and SCITT (>2mm and >4mm) for pastoral cattle sampled in the North West Region and Vina Division**

8.3.3 Factors associated with disagreement between the IFN-gamma assay and SCITT

The potential reasons for disagreement, between the IFN-γ assay and SCITT in pastoral cattle were investigated. Although the level of agreement is similar between the IFN-γ assay and SCITT in both study sites (NWR and VD), the potential reasons for disagreement were investigated separately as these differed between the two study sites in the abattoir study (Chapter 4). MLR models were constructed to investigate the potential combination of factors for diagnostic disagreement between the IFN-γ assay ($\geq$0.1) and SCITT (>2mm). Body condition score (BCS) is not included as an explanatory variable in the models as it is more likely to be a result of being infected by bTB, another disease or differences in time of year cattle were sampled between the study sites (Related to availability of grazing). Potential interactions between sex and age (Using DS) (Figure 4.4) along with breed, *F. gigantica* serology status and treatment with an anthelmintic in the previous 12 months were included in model selection.

IFN-gamma assay positive and SCITT negative

Firstly results from sampled cattle were subsetted to investigate diagnostic disagreement for being IFN-γ assay positive and SCITT negative using two methods to produce two final models per study site:

- **Model a):** Subsetted as IFN-γ assay ($\geq$0.1) positive and using SCITT ($\leq$2mm) negative as the dependent variable (Figure 8.1 refers to table 8.3).
- **Model b):** Subsetted as SCITT ($\leq$2mm) negative and using IFN-γ assay ($\geq$0.1) positive as the dependent variable (Figure 8.1 refers to table 8.3).

The two selected final models constructed, for NWR and VD pastoral cattle, did not identify any statistically significant (p value$\leq$0.05) variables associated with IFN-γ positive and SCITT negative disagreement (Table 8.3).

IFN-gamma assay negative and SCITT positive

Separately for the NWR and VD, pastoral cattle were also subsetted to investigate diagnostic test disagreement for being SCITT positive (>2mm) and IFN-γ assay (<0.1) negative using two methods:

1. **Model c):** Subsetted as IFN-γ assay (<0.1) negative and using SCITT (>2mm) positive as the dependent variable (Figure 8.2 refers to table 8.4).
2. **Model d):** Subsetted as SCITT (>2mm) positive and using IFN-γ assay (<0.1) as the dependent variable (Figure 8.2 refers to table 8.4).

The two selected final models constructed, for NWR and VD pastoral cattle, did not identify any statistically significant (p value$\leq$0.05) variables associated with IFN-γ negative and SCITT positive disagreement (Table 8.3).

IFN-gamma assay positive and SCITT negative			
North West Region			
(a) IFN-gamma assay POSITIVE subgroup (>=0.1, n=85)			
Model	K	AIC	Δ AIC
SCITTdiff2∼CATDEN + ANISEX + ABREED + QUESWM + FgLivB + (1|HER_ID)	7	120.74	6.25
SCITTdiff2∼CATDEN * ANISEX + ABREED * FgLivB + QUESWM + (1|HER_ID)	9	121.01	6.53
SCITTdiff2∼CATDEN * ANISEX + ABREED + FgLivB * QUESWM + (1|HER_ID)	9	121.69	7.20
SCITTdiff2∼CATDEN * ANISEX + ABREED + FgLivB + QUESWM + (1|HER_ID)	8	121.28	6.79
SCITTdiff2∼CATDEN + ANISEX + ABREED * FgLivB + QUESWM + (1|HER_ID)	8	120.42	5.93
SCITTdiff2∼CATDEN + ANISEX + ABREED + FgLivB * QUESWM + (1|HER_ID)	8	120.17	5.68
SCITTdiff2∼1 + (1|HER_ID)	2	114.49	0.00
(b) SCITT NEGATIVE subgroup (>2mm, n=706)			
Model	K	AIC	Δ AIC
bovigam01∼CATDEN + ANISEX + ABREED + QUESWM + FgLivB + (1|HER_ID)	7	377.43	5.58
bovigam01∼CATDEN * ANISEX + ABREED * FgLivB + QUESWM + (1|HER_ID)	9	378.84	8.84
bovigam01∼CATDEN * ANISEX + ABREED + FgLivB * QUESWM + (1|HER_ID)	9	376.10	9.05
bovigam01∼CATDEN * ANISEX + ABREED + FgLivB + QUESWM + (1|HER_ID)	8	379.45	7.00
bovigam01∼CATDEN + ANISEX + ABREED * FgLivB + QUESWM + (1|HER_ID)	8	376.83	7.41
bovigam01∼CATDEN + ANISEX + ABREED + FgLivB * QUESWM + (1|HER_ID)	8	374.16	7.63
bovigam01∼1 + (1|HER_ID)	2	139.60	0.00
Vina Division			
(a) IFN-gamma assay POSITIVE subgroup (>=0.1, n=48)			
Model	K	AIC	Δ AIC
SCITTdiff2∼CATDEN + ANISEX + ABREED + QUESWM + FgLivB + (1|HER_ID)	8	37.48	13.88
SCITTdiff2∼CATDEN * ANISEX + ABREED * FgLivB + QUESWM + (1|HER_ID)	11	46.43	22.83
SCITTdiff2∼CATDEN * ANISEX + ABREED + FgLivB * QUESWM + (1|HER_ID)	10	43.29	19.69
SCITTdiff2∼CATDEN * ANISEX + ABREED + FgLivB + QUESWM + (1|HER_ID)	9	40.08	16.48
SCITTdiff2∼CATDEN + ANISEX + ABREED * FgLivB + QUESWM + (1|HER_ID)	10	43.34	19.74
SCITTdiff2∼CATDEN + ANISEX + ABREED + FgLivB * QUESWM + (1|HER_ID)	9	40.53	16.93
SCITTdiff2∼1 + (1|HER_ID)	2	23.60	0.00
(b) SCITT NEGATIVE subgroup (>2mm, n=716)			
Model	K	AIC	Δ AIC
bovigam01∼CATDEN + ANISEX + ABREED + QUESWM + FgLivB + (1|HER_ID)	8	277.92	0.24
bovigam01∼CATDEN * ANISEX + ABREED * FgLivB + QUESWM + (1|HER_ID)	11	280.12	2.44
bovigam01∼CATDEN * ANISEX + ABREED + FgLivB * QUESWM + (1|HER_ID)	10	279.71	2.03
bovigam01∼CATDEN * ANISEX + ABREED + FgLivB + QUESWM + (1|HER_ID)	9	277.68	0.11
bovigam01∼CATDEN + ANISEX + ABREED * FgLivB + QUESWM + (1|HER_ID)	10	280.16	2.48
bovigam01∼CATDEN + ANISEX + ABREED + FgLivB * QUESWM + (1|HER_ID)	9	279.96	2.29
bovigam01∼1 + (1|HER_ID)	2	277.68	0.00

Table 8.3: **Disagreement model selection to investigate risk factors for pastoral cattle being IFN-gamma assay positive and SCITT negative.**

Each of study site has two models to investigate pastoral cattle being IFN-gamma assay positive and SCITT negative: (a) Dependent variable SCITT negative (SCITTdiff2) in IFN-γ positive sub group. (b) Dependent variable IFN-γ assay positive (bovigam01) in SCITT negative sub group. Explanatory variables included are CATDEN (Age: $\geq$3 years and <3 years), ANISEX (Sex: Female or male), ABREED (Breed: Gudali, mixed breed or Fulani), FgLivB (*F. gigantica* serology result: Negative or positive), QUESWM (Whether treated with anthelmintic in the previous 12 months: Yes or No) and random effect HER_ID (Herd sampled from). Key: Significant selected model= Grey; K= Number of parameters; AIC= Akaike information criterion; Δ AIC= Delta akaike information criterion; *= Interaction between variables.

IFN-gamma assay negative and SCITT positive			
North West Region			
(c) IFN-gamma assay NEGATIVE subgroup (>=0.1, n=665)			
Model	K	AIC	Δ AIC
SCITTdiff2∼CATDEN + ANISEX + ABREED + QUESWM + FgLivB + (1|HER_ID)	7	145.18	5.58
SCITTdiff2∼CATDEN * ANISEX + ABREED * FgLivB + QUESWM + (1|HER_ID)	9	148.44	8.84
SCITTdiff2∼CATDEN * ANISEX + ABREED + FgLivB * QUESWM + (1|HER_ID)	9	148.65	9.05
SCITTdiff2∼CATDEN * ANISEX + ABREED + FgLivB + QUESWM + (1|HER_ID)	8	146.59	7.00
SCITTdiff2∼CATDEN + ANISEX + ABREED * FgLivB + QUESWM + (1|HER_ID)	8	147.01	7.41
SCITTdiff2∼CATDEN + ANISEX + ABREED + FgLivB * QUESWM + (1|HER_ID)	8	147.23	7.63
SCITTdiff2∼1 + (1|HER_ID)	2	139.60	0.00
(d) SCITT POSITIVE subgroup (>2mm, n=44)			
Model	K	AIC	Δ AIC
bovigam01∼CATDEN + ANISEX + ABREED + QUESWM + FgLivB + (1|HER_ID)	7	69.60	69.60
bovigam01∼CATDEN * ANISEX + ABREED * FgLivB + QUESWM + (1|HER_ID)	9	69.17	69.17
bovigam01∼CATDEN * ANISEX + ABREED + FgLivB * QUESWM + (1|HER_ID)	9	69.92	69.92
bovigam01∼CATDEN * ANISEX + ABREED + FgLivB + QUESWM + (1|HER_ID)	8	66.79	9.19
bovigam01∼CATDEN + ANISEX + ABREED * FgLivB + QUESWM + (1|HER_ID)	8	71.88	71.88
bovigam01∼CATDEN + ANISEX + ABREED + FgLivB * QUESWM + (1|HER_ID)	8	72.53	72.53
bovigam01∼1 + (1|HER_ID)	2	57.60	0.00
Vina Division			
(c) IFN-gamma assay NEGATIVE subgroup (>=0.1, n=699)			
Model	K	AIC	Δ AIC
SCITTdiff2∼CATDEN + ANISEX + ABREED + QUESWM + FgLivB + (1|HER_ID)	8	120.10	4.00
SCITTdiff2∼CATDEN * ANISEX + ABREED * FgLivB + QUESWM + (1|HER_ID)	11	116.36	0.26
SCITTdiff2∼CATDEN * ANISEX + ABREED + FgLivB * QUESWM + (1|HER_ID)	10	117.76	1.66
SCITTdiff2∼CATDEN * ANISEX + ABREED + FgLivB + QUESWM + (1|HER_ID)	9	116.69	0.87
SCITTdiff2∼CATDEN + ANISEX + ABREED * FgLivB + QUESWM + (1|HER_ID)	10	119.21	3.12
SCITTdiff2∼CATDEN + ANISEX + ABREED + FgLivB * QUESWM + (1|HER_ID)	9	121.60	5.50
SCITTdiff2∼1 + (1|HER_ID)	2	116.09	0.00
(d) SCITT POSITIVE subgroup (>2mm, n=24)			
Model	K	AIC	Δ AIC
bovigam01∼CATDEN + ANISEX + ABREED + QUESWM + FgLivB + (1|HER_ID)	8	35.51	12.05
bovigam01∼CATDEN * ANISEX + ABREED * FgLivB + QUESWM + (1|HER_ID)	9	40.70	17.24
bovigam01∼CATDEN * ANISEX + ABREED + FgLivB * QUESWM + (1|HER_ID)	9	40.90	17.37
bovigam01∼CATDEN * ANISEX + ABREED + FgLivB + QUESWM + (1|HER_ID)	8	35.52	12.07
bovigam01∼CATDEN + ANISEX + ABREED * FgLivB + QUESWM + (1|HER_ID)	9	40.67	17.22
bovigam01∼CATDEN + ANISEX + ABREED + FgLivB * QUESWM + (1|HER_ID)	9	40.76	17.31
bovigam01∼1 + (1|HER_ID)	2	23.46	0.00

Table 8.4: **Disagreement model selection to investigate risk factors for pastoral cattle being IFN-gamma assay negative and SCITT positive.**

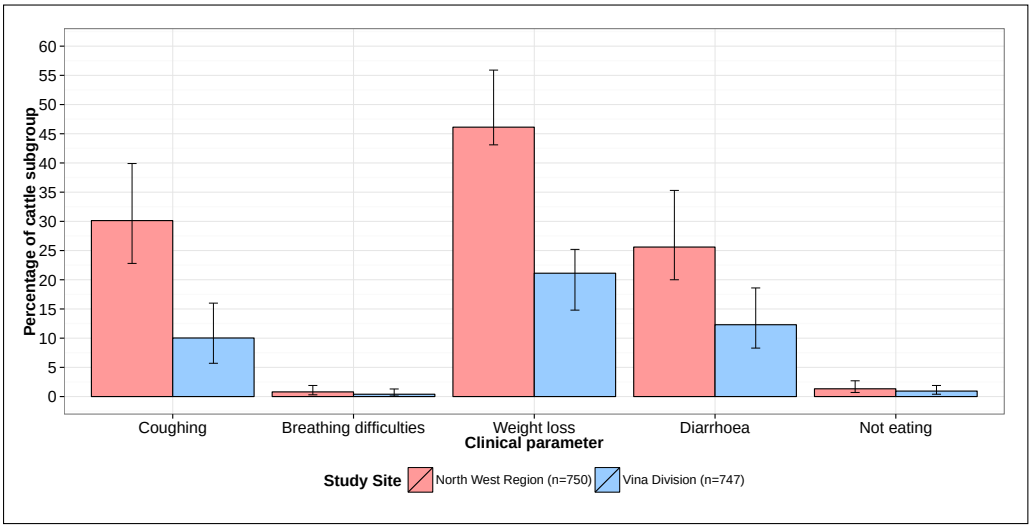
Each of study site has two models to investigate pastoral cattle being IFN-gamma assay negative and SCITT positive: (c) Dependent variable SCITT positive (SCITTdiff2) in IFN-γ negative sub group. (d) Dependent variable IFN-γ assay negative (bovigam01) in SCITT positive sub group. Explanatory variables included are CATDEN (Age: $\geq$3 years and <3 years), ANISEX (Sex: Female or male), ABREED (Breed: Gudali, mixed breed or Fulani), FgLivB (*F. gigantica* serology result: Negative or positive), QUESWM (Whether treated with anthelmintic in the previous 12 months: Yes or No) and random effect HER_ID (Herd sampled from). Key: Significant selected model= Grey; K= Number of parameters; AIC= Akaike information criterion; Δ AIC= Delta akaike information criterion; *= Interaction between variables.

8.3.4 Association of bovine tuberculosis diagnostic test result and reported clinical signs

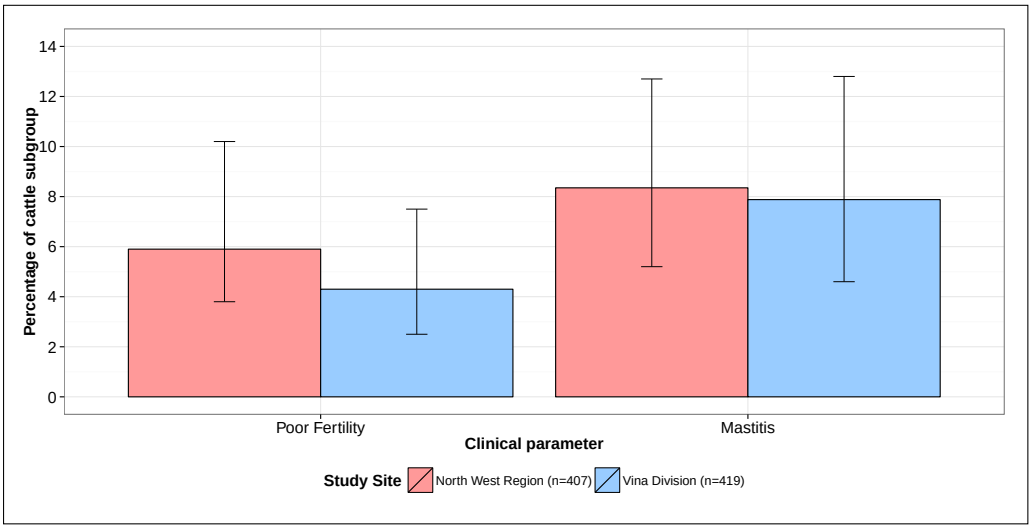
Only clinical sign data from the pastoral cattle sample were analysed as dairy farmers were poor at recognising clinical signs (Chapter 7). Pastoralists reported clinical signs present at the time of sampling except "poor fertility" which was reported if present in the previous 12 months. Pastoralists in the VD mainly reported cattle had no clinical signs of disease (Table 8.5), compared to the NWR where more than half of sampled cattle were reported with one or more clinical signs at the times of sampling. Weight loss and coughing were the most frequently reported clinical signs in both the NWR and VD (Figure 8.6). Weight loss, coughing and diarrhoea were more frequently reported in the NWR than the VD. Correlation matrices demonstrated the strongest correlation between "weight loss and diarrhoea" across all sampled cattle. Less correlated clinical signs were "coughing and weight loss", "coughing and diarrhoea" and "not eating and breathing difficulties". These patterns were also noted in breeding females (Female cattle $\geq$2 years of age) in addition to "mastitis and weight loss" (Figure 8.7). No difference in OR was noted between bTB positive and negative cattle with clinical signs using either IFN-γ assay ($\geq$0.1) or SCITT (>2mm) to define bTB status (Table 8.6).

Number of reported clinical signs	North West Region n=750 (95% CI)	Vina Division n=748 (95% CI)
0	39.2% (32.4-46.5%)	69.7% (61.3-77.0%)
1	25.0% (20.4-30.2%)	16.2% (12.7-20.4%)
2	19.9% (15.2-25.7%)	10.0% (7.2-13.8%)
3 or more	15.9% (10.3-23.7%)	4.1% (2.2-7.5%)

Table 8.5: **Descriptive summary of clinical signs reported in pastoral from cross-sectional study.**

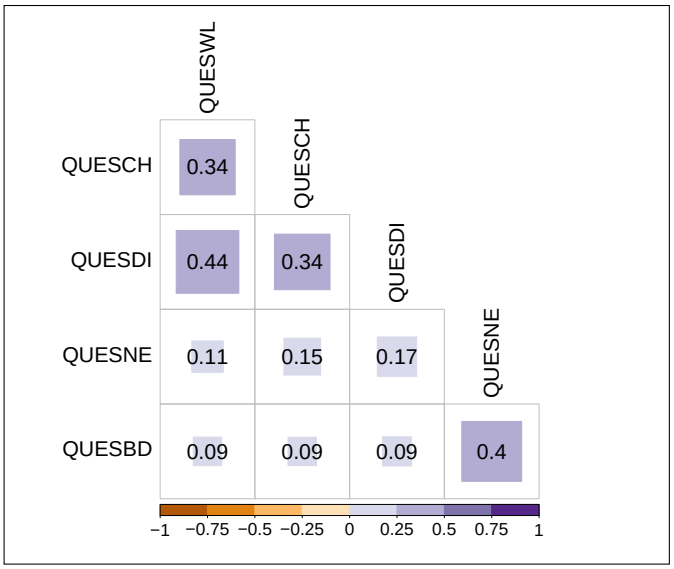


(a) Clinical signs reported in pastoral, in the North West (n=750) and the Vina (n=748), including 95% CI at the time of sampling.

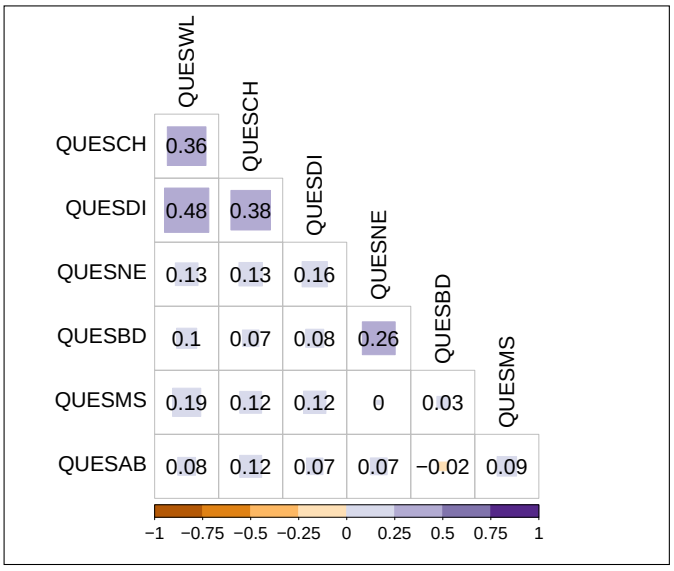


(b) Female $\geq$ 2 years of age only clinical signs reported in pastoral, in the North West (n=388) and Vina (n=410), including 95% CI in the previous 12 months.

Figure 8.6: **Clinical signs in cattle sampled in cross-sectional studies.**



(a) All pastoral cattle (n=1498)



(b) Female pastoral cattle $\geq$ 2 years of age (n=798)

Figure 8.7: **Correlation matrix between clinical signs reported in pastoral cattle.** Purple indicates and positive association and orange indicates a negative correlation. The diameter represents the magnitude of correlation, demoted by r in black. Clinical sign variables include "Weight loss" (QUESWL), "Diarrhoea" (QUESDI), "Not eating" (QUESNE) and "Breathing difficulties" (QUESBD). "Mastitis" (QUESMS) and "Poor fertility" (QUESAB) were included for for female animals only.

Clinical sign		bTB status (IFN-γ assay $\geq$0.1)					bTB status (SCITT >2mm)			
		+	-	OR	95% CI		+	-	OR	95% CI
Pastoral cattle n=1498										
Weight loss (QUESWL)	+	51	453	0.98	0.69- 1.4	+	24	478	1.08	0.65-1.79
	-	102	892			-	44	945		
Coughing (QUESCH)	+	29	272	0.92	0.60-1.41	+	8	293	0.51	0.24-1.08
	-	124	1073			-	60	1130		
Diarrhoea (QUESDI)	+	26	258	0.86	0.55-1.34	+	7	272	0.48	0.22-1.06
	-	127	1087			-	61	1148		
Not eating (QUESNE)	+	1	16	0.55	0.07-4.2	+	1	16	1.31	0.17-10.04
	-	152	1329			-	67	1407		
Breathing difficulties (QUESBD)	+	0	9	0.97	0.12-7.76	+	0	9	2.34	0.29-18.78
	-	153	1336			-	68	1414		
Pastoral breeding female cattle (Female cattle $\geq$2 years old) n=798										
Mastitis (QUESMS)	+	6	61	0.9	0.38-2.15	+	5	62	1.41	0.54-3.69
	-	75	684			-	41	715		
Poor fertility (QUESAB)	+	3	39	0.7	0.21-2.31	+	4	38	1.85	0.63-5.43
	-	78	706			-	42	739		

Table 8.6: **Association of clinical signs with bovine tuberculosis status by IFN-gamma assay ($\geq$0.1) and SCITT (2mm).**

8.3.5 Bovine tuberculosis prevalence in pastoral and dairy cattle.

The estimated bTB prevalences were compared using the IFN-γ assay, SCITT and parallel combination of the two tests. No difference in prevalence was noted between pastoral (NWR and VD) or dairy cattle when using the SCITT (figure 8.8b). For the IFN-γ assay prevalence estimates were higher in dairy cattle (21.7 CI: 13.1-31.6%) and pastoral cattle in the NWR (11.3% CI: 9.3-13.8%) than in VD (6.6% CI: 5.0-8.6%). Single test estimates were similar although the IFN-γ assay had a higher prevalence in NWR than the VD pastoral cattle compared to when using the SCITT alone. Combining test results (Parallel testing; positive on either or both tests) highlighted the higher prevalence in dairy (25.0%, CI: 16.0-37.3%) compared to pastoral cattle in both the NWR (13.2% CI: 11.0-15.8%) and VD (8.1%, CI: 6.3-10.3%). Parallel test estimates were similar for the IFN-γ assay and thus higher than SCITT prevalence estimates.

The IFN-γ assay, SCITT and parallel combination of the two tests were used to estimate subgroup bTB prevalence by age, sex, breed and anthelmintic treatment usage for pastoral, NWR and VD, and dairy cattle (NWR). When investigating prevalence by age, using individual DS values, there is no difference between bTB prevalence between dentition scores using any test combination in pastoral (NWR and VD) and dairy cattle (Figure 8.10). When age was re-catagoried (<3 and $\geq$3 years), a larger proportion of young (<3 years) dairy cattle were IFN-γ positive than young (<3 years) pastoral cattle in the Vina (Table 8.7). Using the SCITT a larger proportion of older ($\geq$3 years) cattle were positive than younger (<3 years) pastoral cattle in the VD.

Fewer female pastoral cattle were IFN-γ positive in the VD than pastoral and dairy

cattle in the NWR. A larger proportion of dairy cattle, that were treated with anthelmintic in the previous 12 months, were IFN-γ positive than pastoral cattle in the VD. A larger proportion of pastoral cattle, which were not treated with anthelmintic in the previous 12 months, were IFN-γ positive in the NWR than VD. Furthermore in the NWR a larger proportion of cattle, that had been treated with anthelmintic in the previous 12 months, were SCITT positive than those that had not been treated in the previous 12 months. Patterns in prevalence between subgroups were similar when using the IFN-γ and parallel testing for pastoral (NWR and VD) and dairy cattle.

Additionally all 1498 cattle from the pastoral cross-sectional study were tested using the *F. gigantica* antibody ELISA. Exposure appears to be endemic in Cameroon with prevalence higher in the VD (58.7% CI: 51.7-65.8%) than the NWR (44.8% CI: 38.5-51.1%). More young (<3 years) pastoral cattle were *F. gigantica* seropositive than young dairy cattle. A larger proportion of old ($\geq$3 years) pastoral cattle were *F. gigantica* seropositive than young (<3 years) pastoral and dairy cattle in the NWR. A larger proportion of female pastoral cattle in the VD were *F. gigantica* seropositive than female pastoral and dairy cattle in the NWR. Furthermore a larger proportion of pastoral mixed breed cattle in the VD were *F. gigantica* seropositive than in the NWR. In the NWR a larger proportion of pastoral cattle, that had been treated with anthelmintic in the previous 12 months, were *F. gigantica* seropositive than dairy cattle.

The prevalence of bTB (using the IFN-γ positivity) and *F. gigantica* seropositivity were compared for pastoral cattle by sub-location in the NWR (by Division) and VD (by Sub-division) to assess for an association between prevalences (Figure 8.11). The figure suggests there to be an inverse association between prevalence of *F. gigantica* infection and bTB by sub-location, although this was not statistically significant (Spearman's correlation coefficient= -0.32, p value=0.24). However, as

seroprevalence of *F. gigantica* at herd level (n=100) was 100% (At least one animal infected per herd) the potential impact on IFN-γ assay test sensitivity was investigated. The assumption was made that all cattle could be exposed to *F. gigantica* to estimate the potential maximum effect of *F. gigantica* co-infection on bTB prevalence, using the IFN-γ assay test sensitivities estimated in chapter 5. There was no difference between estimated (SE= 51.7%) and true bTB prevalence (Figure 8.12). Although when the potential reduction of IFN-γ assay sensitivity (SE= 31.4%), with *F. gigantica* co-infection, was taken into account the apparent prevalence was much lower than the true prevalence estimate in the NWR.

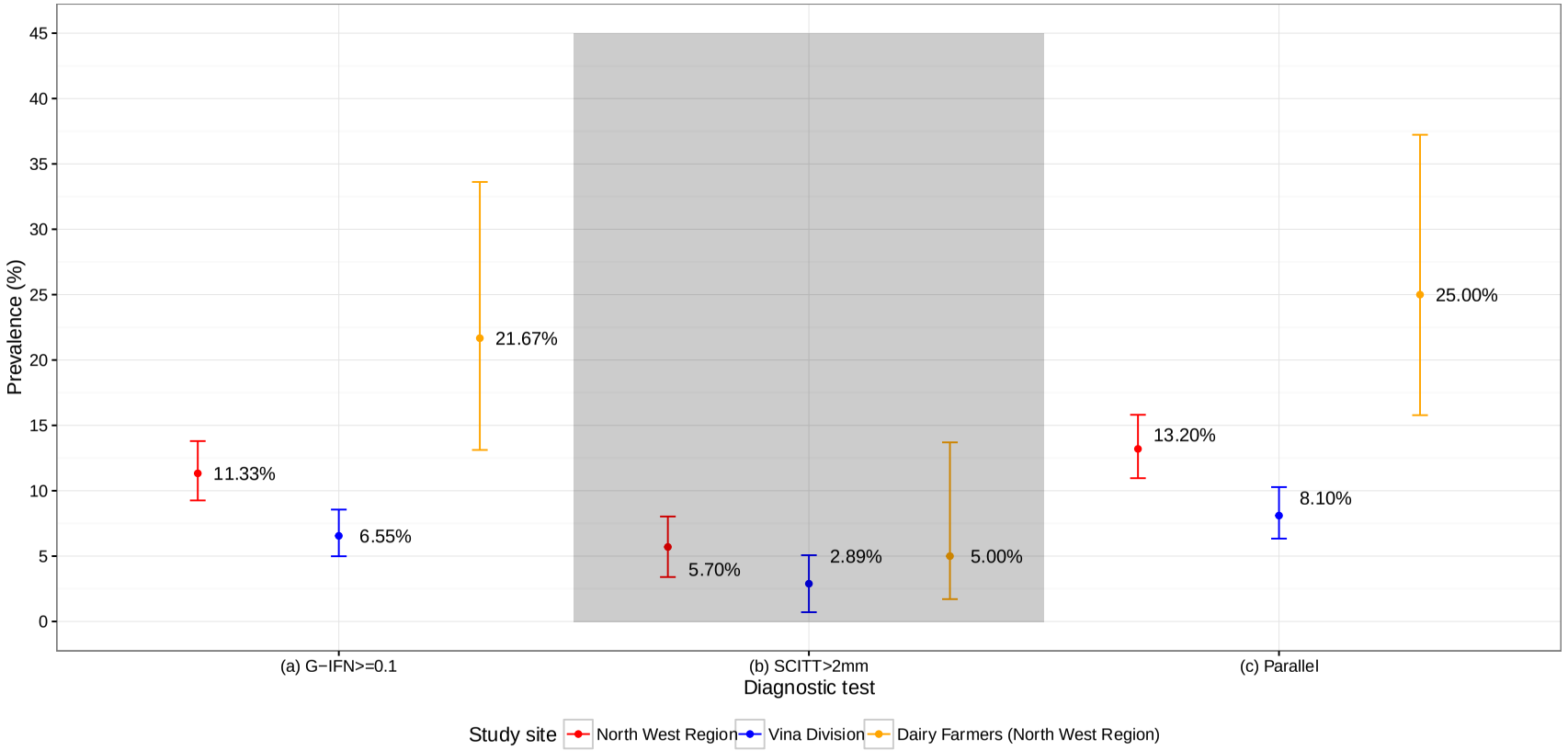
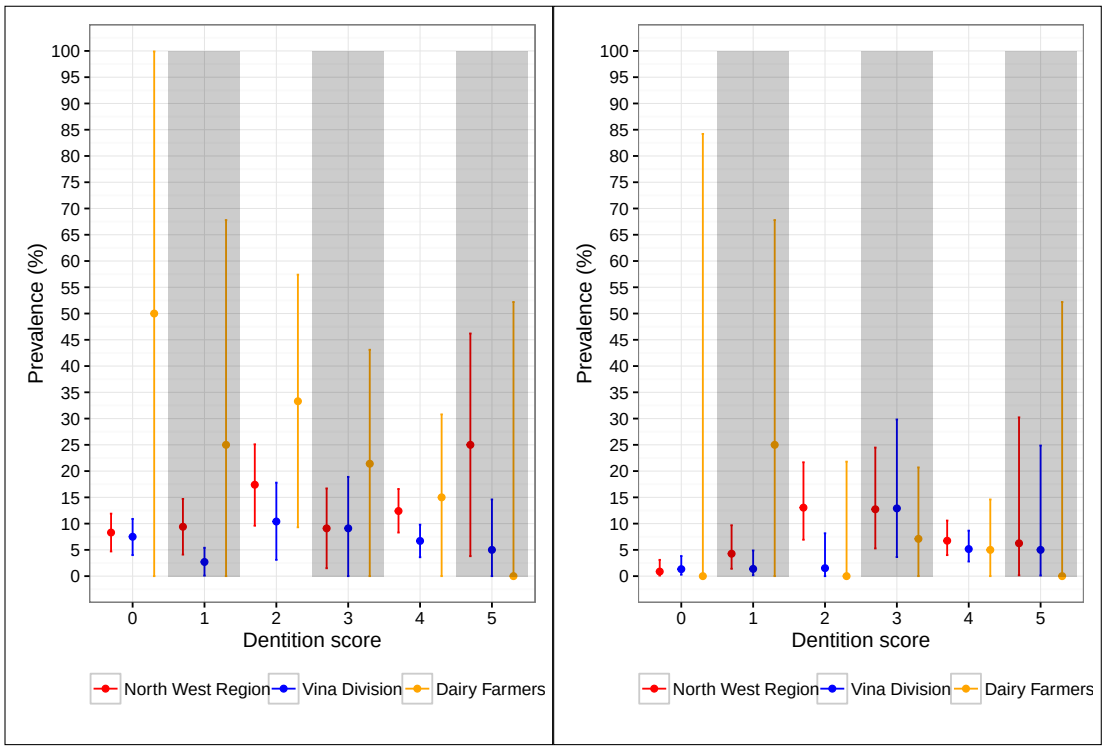


Figure 8.8: **Summary of bTB prevalence, using IFN-gamma assay (≥ 0.1), SCITT and parallel testing, in pastoral and dairy cross-sectional studies.**

IFN-γ assay (≥ 0.1) (NWR n=750; VD n=748; Dairy =60); SCITT (>2mm) and Parallel (NWR n=750; VD n=741; Dairy =60).

(a) **IFN-γ assay** (≥ 0.1)

(b) **SCITT** (>2mm)



(c) **Parallel**

(d) ***F. gigantica* serology**

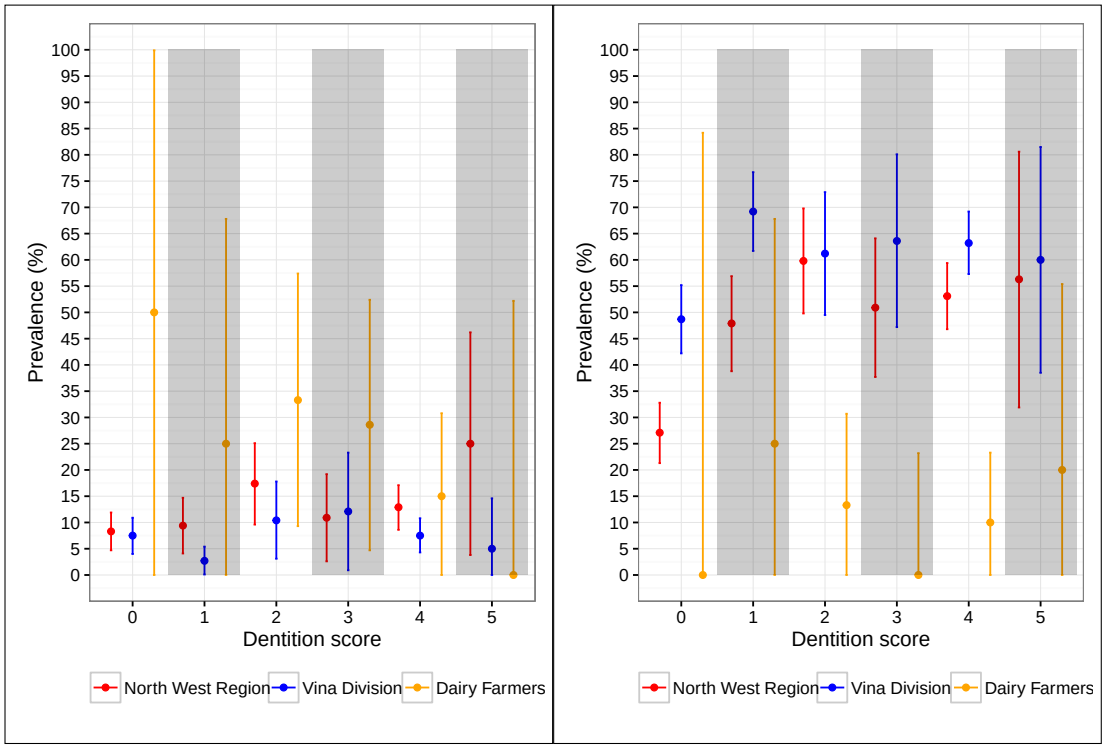


Figure 8.10: **Prevalence by various diagnostic tests by dentition score for pastoral (NWR and VD) and dairy cattle.**

IFN-γ assay (≥ 0.1) and *F. gigantica* serology (NWR n=750; VD n=748; Dairy =60); SCITT (>2mm) and Parallel (NWR n=750; VD n=741; Dairy =60).

Subsets	Pastoral cattle				Dairy cattle	
	North West Region		Vina Division		North West Region	
	IFN-gamma	n	IFN-gamma	n	IFN-gamma	n
< 3 years	10.5% (7.6-13.4%)	750	6.3% (4.1-8.6%)	748	33.3% (13.0-53.7%)	60
>= 3 years	12.5% (8.8-16.2%)		6.8% (4.0-9.7%)		15.4% (4.0-26.8%)	
Female	11.2% (8.5-13.9%)	750	5.8% (3.8-7.7%)	748	22.0% (12.3-34.7%)	60
Male	11.7% (7.5-15.9%)		8.6% (4.8-12.4%)		0.0% (0.0-97.5%)	
Mixed breed	12.2% (9.3-15.1%)	750	11.2% (5.2-17.2%)	748	[redacted]	60
Fulani	9.8% (6.2-13.4%)		8.3% (5.2-17.2%)		[redacted]	
Gudali	[redacted]		5.7% (3.8-7.5%)		[redacted]	
Holstein-Friesian	[redacted]		[redacted]		21.7% (12.1-34.2%)	
Treated with anthelmintic	8.7% (5.8-11.6%)	750	5.9% (2.8-9.0%)	748	22.2% (11.1-33.4%)	60
Not treated with anthelmintic	13.7% (10.3-17.1%)		6.7% (4.5-8.8%)		25.0% (0.0-67.8%)	
Subsets	North West Region		Vina Division		North West Region	
	SCITT	n	SCITT	n	SCITT	n
< 3 years	4.3% (2.4-6.2%)	750	1.4% (0.3-2.5%)	741	4.8% (0.0-13.9%)	60
>= 3 years	8.0% (5.0-11.0%)		5.9% (3.3-8.6%)		5.1% (0.0-12.1%)	
Female	5.9% (3.9-7.9%)	750	3.8% (2.1-5.4%)	741	5.0% (1.0-13.9%)	60
Male	5.9% (2.8-8.9%)		1.9% (0.01-3.8%)		0.0% (0.0-97.5%)	
Mixed breed	6.0% (3.9-8.1%)	750	7.5% (2.5-12.5%)	741	[redacted]	60
Fulani	5.7% (2.9-8.4%)		12.5% (0.0-25.7%)		[redacted]	
Gudali	[redacted]		2.1% (1.0-3.3%)		[redacted]	
Holstein-Friesian	[redacted]		[redacted]		5.0% (1.0-13.9%)	
Treated with anthelmintic	3.1% (1.3-4.9%)	750	1.4% (0.0-2.9%)	748	3.7% (0.0-8.8%)	60
Not treated with anthelmintic	8.4% (5.7-11.1%)		4.0% (2.3-5.7%)		6.3% (1.2-15.6%)	

Table 8.7: **Summary of IFN-gamma assay (≥ 0.1) and SCITT (>2mm) positivity in pastoral and dairy cross-sectional studies by age (AN-IDEN by DS), sex (ANISEX), breed (ABREED) and history of anthelmintic treatment in the previous 12 months (QUESWM).**
IFN-γ assay and *F. gigantica* antibody ELISA (≥ 0.1) (NWR n=750; VD n=748; Dairy =60); SCITT (>2mm) and Parallel (NWR n=750; VD n=741; Dairy =60).

Subsets	North West Region		Vina Division		North West Region	
	Parallel	n	Parallel	n	Parallel	n
< 3 years	10.5% (7.6-13.4%)	750	6.3% (4.1-8.6%)	748	33.3% (13.0-53.7%)	60
>= 3 years	13.1% (9.4-16.9%)		7.8% (4.8-10.8%)		15.4% (4.0-26.8%)	
Female	11.4% (8.7-14.1%)	750	6.1% (4.1-8.2%)	748	23.7% (13.6-36.6%)	60
Male	12.2% (7.9-16.5%)		9.0% (5.2-12.9%)		0.0% (0.0-97.5%)	
Mixed breed	12.2% (9.3-15.1%)	750	12.2% (6.0-18.3%)	748	[redacted]	60
Fulani	9.8% (6.2-13.4%)		12.5% (6.0-18.3%)		[redacted]	
Gudali	[redacted]		5.8% (4.0-7.7%)		[redacted]	
Holstein-Friesian	[redacted]		[redacted]		23.3% (13.4-36.0%)	
Treated with anthelmintic	9.0% (6.0-11.9%)	750	5.9% (2.8-9.0%)	748	22.2% (11.0-33.4%)	60
Not treated with anthelmintic	14.0% (10.6-17.4%)		7.2% (5.0-9.4%)		50.0% (0.5-99.4%)	
Subsets	North West Region		Vina Division		North West Region	
	F. gigantica serology	n	*F. gigantica* serology	n	*F. gigantica* serology	n
< 3 years	39.5% (34.9-44.1%)	750	57.4% (52.8-62.0%)	748	14.3% (0.0-29.4%)	60
>= 3 years	52.9% (47.3-58.4%)		62.9% (57.5-68.3%)		7.7% (0.0-16.1%)	
Female	47.3% (43.1-51.6%)	750	61.0% (56.8-65.1%)	748	10.0% (3.8-20.8%)	60
Male	39.6% (33.2-46.1%)		56.2% (49.5-62.9%)		0.0% (0.0-97.5%)	
Mixed breed	44.5% (40.1-49.0%)	750	62.6% (53.4-71.8%)	748	[redacted]	60
Fulani	46.0% (40.0-52.0%)		58.33% (38.6-78.1)		[redacted]	
Gudali	[redacted]		59.2% (55.3-63.0%)		[redacted]	
Holstein-Friesian	[redacted]		[redacted]		10.0% (3.8-20.8%)	
Treated with anthelmintic	42.6% (37.4-47.7%)	750	52.5% (45.9-59.1%)	748	9.3% (1.5-17.1%)	60
Not treated with anthelmintic	47.3% (42.4-52.3%)		62.5% (58.4-66.7%)		25.0% (0.0-67.8%)	

Table 8.8: **Summary of parallel test and *F. gigantica* antibody ELISA positivity in pastoral and dairy cross-sectional studies by age (ANIDEN by DS), sex (ANISEX), breed (ABREED) and history of anthelmintic treatment in the previous 12 months (QUESWM).**
IFN-γ assay and *F. gigantica* antibody ELISA ($\geq$ 0.1) (NWR n=750; VD n=748; Dairy =60); SCITT (>2mm) and Parallel (NWR n=750; VD n=741; Dairy =60).

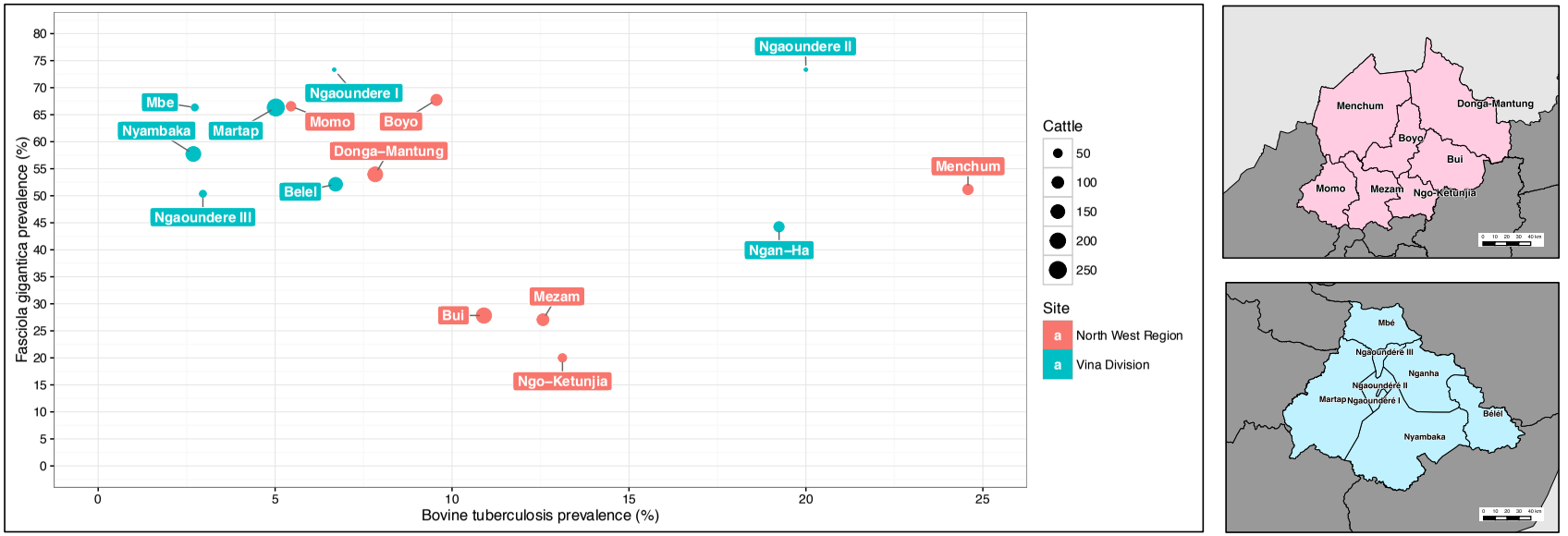


Figure 8.11: **Comparison of bovine tuberculosis (IFN-gamma assay) and *F. gigantica* (Serology) prevalence by sub-location in the North West Region (Division) and Vina Division (Sub-division).**
North West Region (Red; n=750) and Vina Division (Blue; n=748). Size of point relative to number of cattle sampled per sub-location with name of sub-location attached.

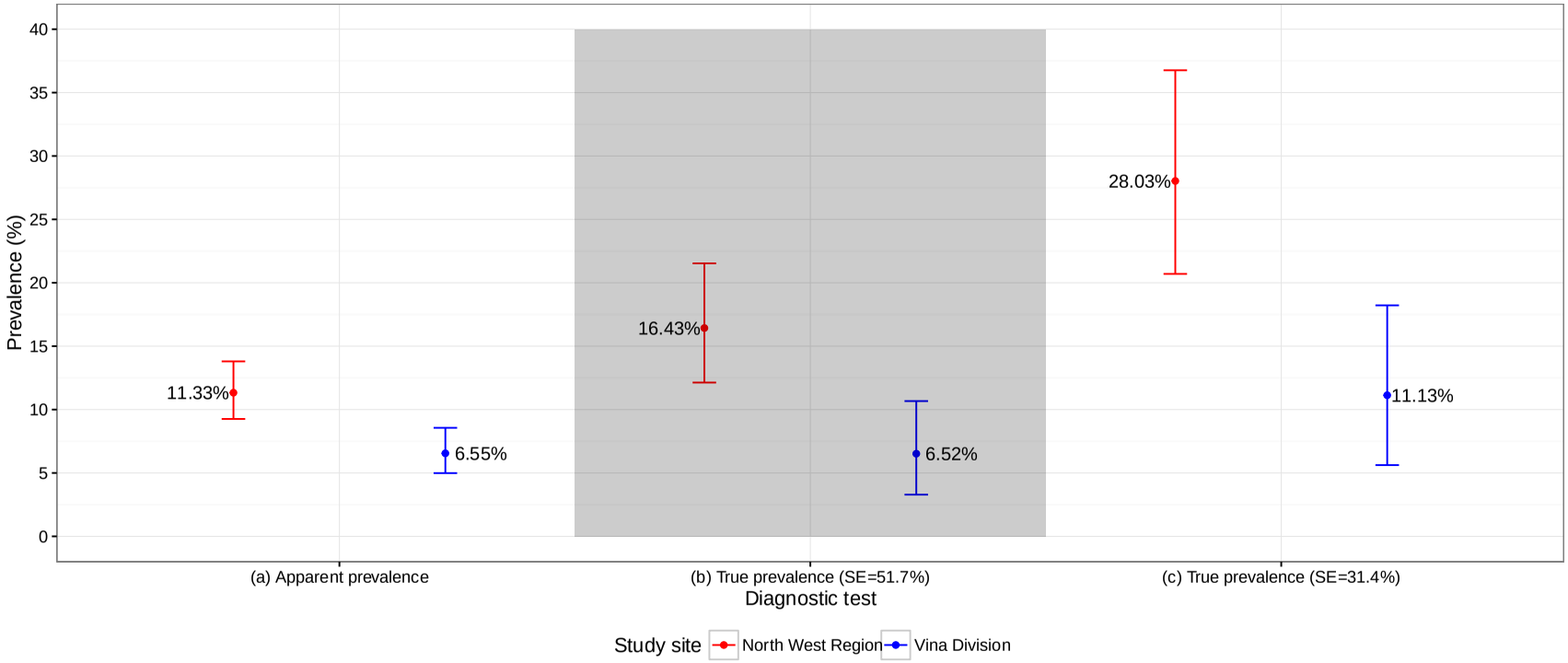


Figure 8.12: **Summary of bTB apparent and true prevalence, assuming sensitivity of the IFN-gamma assay was 51.7% and 31.4% respectively, in pastoral cattle.**

North West Region (Red; n=750) and Vina Division (Blue; n=748).

8.3.6 Potential risk factors for IFN-gamma positivity, including *F. gigantica* status, in pastoral and dairy cattle

The potential influence of *F. gigantica* exposure (*F. gigantica* seropositivity) on IFN-γ positivity in pastoral cattle was further investigated. Using the IFN-γ result to define bTB status, a bTB risk factor MLR model was constructed taking into account other factors which may influence IFN-γ positivity. The model was then constructed with and without *F. gigantica* status, determined by the *F. gigantica* antibody ELISA, to see if *F. gigantica* seropositivity associated with IFN-γ status. Thus pastoral cattle from the NWR and VD were included in a single dataset to specifically investigate the influence of *F. gigantica* seropositivity (n=1498) to increase the overall sample size. A similar MLR model was constructed for dairy cattle (n=60) to compare the potential differences in risk factors between dairy and pastoral cattle.

Pastoral cattle

For the pastoral cattle model selection, study site (strata1; NWR and VD) was included as a fixed effect and herd (HER_ID) was included as a random effect to account for clustering. Univariate analysis identified 15 variables to be included in the pastoral cattle MLR model selection with a p value $\leq$0.2 (Appendix J). All variables were included in the MLR model selection as none were correlated with another. Backwards stepwise model selection was conducted including the final model with each variable removed (table 8.9).

Five risk factors were associated with IFN-γ positivity. Risk factors included number of cattle presented at sampling (NUMCTPc; 50+ cattle; OR: 3.11 CI: 1.20-8.02).

Variables which were protective included sex (ANISEX; Female; OR: 0.38 CI: 0.21-0.70), number of other herds grazed with on a daily basis (GRZNHD; Yes; OR: 0.31 CI: 0.12-0.82), grazing with antelope (GRZANT; Yes; OR: 0.58 CI: 0.34-0.99) and drinking from streams (DRKSTR; Yes; OR: 0.08 CI: 0.01-0.41). Although not significant, age (ANIDEN; $\geq$3; OR: 1.20 CI: 0.60-2.42) and previous treatment with a trypanocide (TRYPRV; No; OR: 1.67 CI: 0.97-2.85) remained in the final model. Including *F. gigantica* serology result did not increase the significance of risk factors in the model and reduced the fit of the final model (Table 8.10).

Dairy cattle

Univariate analysis identified 5 variables to be included in dairy cattle MLR model selection with a p value $\leq$0.2 (Appendix J) with none being correlated. Table 8.9 demonstrates backwards stepwise model selection with the final model (highlighted in grey). Sub-division of co-operative (Bamenda 1, Santa and Jakiri) was included as a fixed effect and herd (HER_ID) was included as a random effect to account for clustering. Cattle from Jakiri dairy cooperative (SUBDIV; Jakiri; OR: 7.86 CI: 1.52-49.22) were significantly at higher odds of bTB positive cattle. Number of cattle presented (NUMCTPc; 3-4 cattle; OR: 0.47 CI: 0.11-2.06. 5-6 cattle; OR: 8.81 CI: 0.99-78.67) was not significant but did improve the fit of the final model (table 8.12).

Assessment of the pastoral and dairy MRL models suggested good fit to explain the dataset. Receiver-operating-curve analysis of pastoral (AUC: 0.81) and dairy (AUC: 0.80) MLR models demonstrated good prediction of the dataset and the residuals of both models appeared to be normally distributed (Histograms not shown).

Model	K	AIC	ΔAIC
bovigam01∼NUMCTPc + ABREED + ANIDEN + ANISEX + SHEEPO + HORSEO + TRYPRV + GRZNHD + GRZANT + FENCEH + DRKWTT + DRKSTR + DRKSAM + SALCAT + strata1 + (1|HER_ID)	23	900.22	17.26
bovigam01∼NUMCTPc + ANIDEN + ANISEX + SHEEPO + HORSEO + TRYPRV + GRZNHD + GRZANT + FENCEH + DRKWTT + DRKSTR + DRKSAM + SALCAT + strata1 + (1|HER_ID)	20	895.97	13.01
bovigam01∼NUMCTPc + ANIDEN + ANISEX + SHEEPO + HORSEO + TRYPRV + GRZNHD + GRZANT + FENCEH + DRKWTT + DRKSTR + SALCAT + strata1 + (1|HER_ID)	17	892.25	9.29
bovigam01∼NUMCTPc + ANIDEN + ANISEX + SHEEPO + HORSEO + TRYPRV + GRZNHD + GRZANT + DRKWTT + DRKSTR + SALCAT + strata1 + (1|HER_ID)	15	889.64	6.68
bovigam01∼NUMCTPc + ANIDEN + ANISEX + SHEEPO + TRYPRV + GRZNHD + GRZANT + DRKWTT + DRKSTR + SALCAT + strata1 + (1|HER_ID)	13	887.22	4.25
bovigam01∼NUMCTPc + ANIDEN + ANISEX + SHEEPO + TRYPRV + GRZNHD + GRZANT + DRKWTT + DRKSTR + strata1 + (1|HER_ID)	12	885.33	2.37
bovigam01∼NUMCTPc + ANIDEN + ANISEX + SHEEPO + TRYPRV + GRZNHD + GRZANT + DRKSTR + strata1 + (1|HER_ID)	11	884.12	1.15
bovigam01∼NUMCTPc + ANIDEN + ANISEX + TRYPRV + GRZNHD + GRZANT + DRKSTR + strata1 + (1|HER_ID)	10	883.56	0.59
bovigam01∼NUMCTPc + ANIDEN*ANISEX + TRYPRV + GRZNHD + GRZANT + DRKSTR + strata1 + (1|HER_ID)	11	882.96	0.00
bovigam01∼ANIDEN*ANISEX + TRYPRV + GRZNHD + GRZANT + DRKSTR + strata1 + (1|HER_ID)	9	885.43	2.47
bovigam01∼NUMCTPc + ANISEX + TRYPRV + GRZNHD + GRZANT + DRKSTR + strata1 + (1|HER_ID)	9	891.34	8.38
bovigam01∼NUMCTPc + ANIDEN + TRYPRV + GRZNHD + GRZANT + DRKSTR + strata1 + (1|HER_ID)	9	889.38	6.42
bovigam01∼NUMCTPc + ANIDEN*ANISEX + GRZNHD + GRZANT + DRKSTR + strata1 + (1|HER_ID)	10	884.27	1.31
bovigam01∼NUMCTPc + ANIDEN*ANISEX + TRYPRV + GRZANT + DRKSTR + strata1 + (1|HER_ID)	10	886.06	3.1
bovigam01∼NUMCTPc + ANIDEN*ANISEX + TRYPRV + GRZNHD + DRKSTR + strata1 + (1|HER_ID)	10	884.86	1.9
bovigam01∼NUMCTPc + ANIDEN*ANISEX + TRYPRV + GRZNHD + GRZANT + strata1 + (1|HER_ID)	10	889.51	6.55

Table 8.9: **Backwards stepwise selection, using delta AIC, multivariate analysis model selection for bTB risk factors using the IFN-gamma assay in pastoral cattle (n=1498).** Dependent variable IFN-γ (bovigam01) positive (1) or negative (0). Explanatory variables included number of cattle presented to represent herd size (NUMCTPc), breed (ABREED), age (ANIDEN), sex (ANISEX), sheep kept with cattle in the previous 12 months (SHEEPO), horses kept with cattle in the previous 12 months (HORSEO), treated with a trypanocide in the previous 12 months (TRYPRV), number of other herds grazed with on a daily basis (GRZNHD), grazed with antelope in the previous 12 months (GRZANT), Fenced in at night (FENCEH), cattle drink mainly from water troughs in the previous 12 months (DRKWTT), cattle drink mainly from streams in the previous 12 months (DRKSTR), number of other herds using watering points (DRKSAM) and selling cattle in the previous 12 months (SALCAT). Clustering was accounted for as herd (HER_ID) as a random effect and study site (strata1; NWR and VD) as a fixed effect. Selected model highlighted in grey. Explanatory variable interaction= *, K= Number of parameters, AIC= Akaike information criterion and Δ AIC= Delta akaike information criterion.

bTB risk factor model for pastoral cattle (n=1498)					bTB risk factor model for pastoral cattle (n=1498)				
A. Without *F. gigantica* serology (AIC: 882.96)					B. With *F. gigantica* serology (AIC: 884.20)				
Variables	**Levels**	**Odds ratio**	**95% CI**	**p value**	**Variables**	**Levels**	**Odds ratio**	**95% CI**	**p value**
strata1	NWR	1			strata1	NWR	1		
	VD	0.76	0.42-1.39	0.39		VD	0.77	0.42-1.41	0.4
NUMCTPc	0-25 cattle	1			NUMCTPc	0-25 cattle	1		
	25-50 cattle	1.94	0.77-4.93	0.16		25-50 cattle	1.96	0.77-4.98	0.15
	50+ cattle	3.11	1.20-8.02	0.02		50+ cattle	3.09	1.19-7.98	0.02
ANIDEN	< 3 years	1			ANIDEN	< 3 years	1		
	>= 3 years	1.20	0.60-2.42	0.61		>= 3 years	1.22	0.60-2.46	0.58
ANISEX	Male	1			ANISEX	Male	1		
	Female	0.38	0.21-0.70	<0.01		Female	0.38	0.21-0.71	<0.01
TRYPRV	Yes	1			TRYPRV	Yes	1		
	No	1.66	0.97-2.85	0.06		No	1.66	0.97-2.84	0.06
GRZNHD	No	1			GRZNHD	No	1		
	Yes	0.31	0.12-0.82	0.02		Yes	0.31	0.12-0.82	0.02
GRZANT	No	1			GRZANT	No	1		
	Yes	0.58	0.34-0.99	0.04		Yes	0.60	0.35-0.99	0.04
DRKSTR	No	1			DRKSTR	No	1		
	Yes	0.08	0.01-0.41	<0.01		Yes	0.07	0.01-0.40	<0.01
ANIDEN:ANISEX	>= 3 years: Female	2.06	0.85-4.98	0.11	ANIDEN:ANISEX	>= 3 years: Female	2.07	0.86-5.00	0.11
					FgLivB	No	1		
						Yes	0.86	0.58-1.28	0.46

Table 8.10: **Final pastoral cattle bTB risk factor multivariate model compared with and without *F. gigantica* serology (FgLivB) (n=1498).** Dependent variable IFN-γ (bovigam01) positive (1) or negative (0). Explanatory variables included number of cattle presented to represent herd size (NUMCTPc), age (ANIDEN), sex (ANISEX), treated with a trypanocide in the previous 12 months (TRYPRV), number of other herds grazed with on a daily basis (GRZNHD), grazed with antelope in the previous 12 months (GRZANT) and cattle drink mainly from streams in the previous 12 months (DRKSTR). Clustering was accounted for as herd (HER_ID) as a random effect and study site (strata1; NWR and VD) as a fixed effect.

bTB risk factor model selection for dairy cattle (n=60)			
Model	**K**	**AICc**	**∆AIC**
bovigam01∼SUBDIV + NUMCTPc + SHEEPO + HOUSEC + BUYCAT +(1|HER_ID)	10	77.33	7.20
bovigam01∼SUBDIV + NUMCTPc + HOUSEC + BUYCAT +(1|HER_ID)	9	74.50	4.37
bovigam01∼SUBDIV + NUMCTPc + BUYCAT +(1|HER_ID)	7	70.87	0.74
bovigam01∼SUBDIV + NUMCTPc +(1|HER_ID)	6	70.13	0.00
bovigam01∼NUMCTPc +(1|HER_ID)	6	71.26	1.13
bovigam01∼SUBDIV +(1|HER_ID)	4	71.26	1.13
bovigam01∼1 +(1|HER_ID)	2	73.80	3.67

Table 8.11: **Backwards stepwise selection, using delta AIC, multivariate analysis model selection for bTB risk factors using the IFN-gamma assay in dairy cattle (n=60).** Dependent variable IFN-γ (bovigam01) positive (1) or negative (0). Explanatory variables included number of cattle presented to represent herd size (NUMCTPc), sheep kept with cattle in the previous 12 months (SHEEPO), dairy cooperative (SUBDIV), cattle housed the majority of the time in the previous 12 months and buying cattle in the previous 12 months (BUYCAT). Clustering was accounted for as herd (HER_ID) as a random effect. Selected model highlighted in grey. K= Number of parameters, AIC= Akaike information criterion and Δ AIC= Delta akaike information criterion.

bTB risk factor model for dairy cattle (n=60)				
Variables	**Levels**	**Odds ratio**	**95% CI**	**p value**
SUBDIV	Bamenda	1		
	Jakiri	7.86	1.25-49.22	0.03
	Santa	0.78	0.11-5.46	0.80
NUMCTPc	1-2	1		
	3-4	0.47	0.11-2.06	0.32
	5-6	8.81	0.99-78.67	0.05

Table 8.12: **Final dairy cattle bTB risk factor multivariate model (n=60).** Dependent variable IFN-γ (bovigam01) positive (1) or negative (0). Explanatory variables included number of cattle presented to represent herd size (NUMCTPc) and dairy cooperative (SUBDIV). Clustering was accounted for as herd as a random effect (HER_ID) and dairy cooperative (SUBDIV).

8.4 Discussion

Clinical signs attributable to bTB were common yet there was no difference in odds of having a clinical sign in bTB positive and negative cattle. Development of clinical signs is partly dependent upon tissue affected by bTB, the extent of lesion pathology and degree of loss of tissue function (18; 436). Hence clinical signs can be present in chronic *M. bovis* infections when pathology is extensive, particularly in the respiratory tract. But cattle can remain outwardly healthy despite extensive systemic disease (437). Also clinical signs associated with bTB are not specific and may be related to other infectious diseases present in Cameroon such as CBPP and gastrointestinal nematodes (438; 214). Despite pastoralists being relatively good at identifying clinical signs of bovine disease (Chapter 7 and (411)) other differential diseases cannot be ruled out without further diagnostic testing on collected serum samples. Although bTB clinical signs may be useful in highlighting the potential presence of chronic bTB, estimating bTB prevalence is dependent upon use of additional diagnostic tests.

This is the first time bTB prevalence has been estimated in Cameroon using the IFN-γ assay. Prevalence was higher in NWR pastoral (11.33% CI: 9.26-13.80%) and dairy cattle (21.67% CI: 13.12-33.62%) compared to pastoral cattle in the VD (6.55% CI: 4.99-8.56%). Bovine tuberculosis prevalence estimates using the IFN-γ assay did not differ from SCITT estimates, and thus the IFN-γ assay could be as useful as the SCITT to detect bTB positive cattle in this setting. Previously the SCITT has been used to assess the prevalence of bTB by Ndukum and others in 2009-10 reporting a higher prevalence in the NWR (22.95%) than the VD (0.55%) (120). In the current study bTB prevalence estimates, using the IFN-γ assay and taking *F. gigantica* co-infection into account, were similar in pattern to the Ndukum et al 2012 study. However, in the current study the SCITT gave lower prevalence estimates in the NWR

and a similar prevalence in the VD to the Ndukum et al 2012 study. The higher prevalence, reported by Ndukum et al 2012, may be down to differences in study design as dairy cattle were also sampled and subsequently might have elevated prevalence estimates compared to the current study.

The IFN-γ assay had best agreement with the SCITT using the $\geq$0.1 and >2mm positive cut-offs respectively (κ= 0.42 (moderate agreement (0.41-0.60)). A degree of agreement is likely between the IFN-γ assay and the SCITT as both measure the CMI response to *M. bovis* (164). Lower agreement between the IFN-γ assay and the SCITT was reported at >4mm. Poor agreement (κ0.13) has also been reported between the two assays in Ethiopia where the >4mm cut-off was used for the SCITT and $\geq$0.1 for the IFN-γ assay (439). Other studies in SSA, including Cameroon, reported that bTB positive *B. indicus* cattle respond differently to *M. bovis* PPD, when compared to *B. taurus* cattle, with improved agreement when using the >2mm cut-off for the SCITT (440; 373; 189). Hence use of a lower >2mm cut-off for the SCITT could be more appropriate in Cameroon.

Being IFN-γ positive and SCITT negative was the most common form of disagreement in this study and potentially associated with the low sensitivity of the SCITT as reported in other studies (368; 370; 186; 433; 441), although specific factors which influenced disagreement were not identified. Less than 100% agreement is not necessarily surprising taking into account variation in immune responses and it is reported that the IFN-γ assay and the SCITT do not necessarily identify the same bTB positive population of cattle (181; 396). In this study cattle were likely infected with *M. bovis* at different time points and subsequently likely to be at different stage in pathogenesis at time of sampling (Chapter 4). The IFN-γ assay can detect *M. bovis* infection weeks-months earlier than the SCITT test and so not all IFN-γ assay positives/ SCITT negatives are necessarily false positives (264; 86).

Cattle with chronic *M. bovis* infections are thought to be more likely to become SCITT positive if they initially start out as IFN-γ positive (371; 85; 370). Non-tuberculous mycobacteria have been shown to infect Cameroonian cattle (379) yet as both CMI diagnostic tests included a control avian PPD in their protocols the impact of NTMs is likely limited on disagreement between the two bTB diagnostics (163; 78; 386) (chapter 4).

Negative IFN-γ assay/ positive SCITT disagreement was less frequently reported which is potentially due to the higher specificity of the SCITT (368; 370; 186; 441; 433). Additionally, IFN-γ responses can fluctuate throughout the course of *M. bovis* infection and may lead to IFN-γ assay false negatives(161; 433) but it is unclear why and further investigations are required on variation in the immune responses in naturally infected cattle.

Fasciola gigantica co-infection has the potential to contribute to the underestimation of bTB prevalence if the sensitivity of the IFN-γ assay is much lower than published estimates (Chapter 5) leading to false negative results. Hence the apparent IFN-γ prevalence estimates in this study could be underestimating the true prevalence in Cameroon due to *F. gigantica* co-infections. When the reduced sensitivity of the IFN-γ assay was taken into account, with exposure to *F. gigantica* being 100% across pastoral herds, prevalence estimates in pastoral cattle were higher (NWR= 28.03% CI: 20.70-36.76%; VD= 11.34% CI: 5.2-18.22%) than apparent prevalence estimates in the NWR (NWR= 11.33% CI: 9.26-13.80%; VD= 6.55% CI: 4.99-8.56%) and subsequently co-infection interaction should be considered when estimating bTB prevalence in Cameroon. However *F. gigantica* exposure was not identified as a risk factor for bTB in multivariate analysis. The main reason is likely that *Fasciola* serology only measures exposure rather than active infection so may not detect current co-infection. Using a *Fasciola* diagnostic, such as FWEC or *Fasciola*

copro-antigen ELISA, may have been more useful to investigate current co-infection interactions. As presence of *Fasciola* parasites, rather than previous exposure, is likely needed for alterations in immune response (297; 293). It is also unclear how early in infection the ELISA can detect parasites or what burden of parasites so this might affect the sensitivity of the *F. gigantica* ELISA. *Fasciola* species infections may affect the detection of bTB positive cattle, using the IFN-γ assay, differently at different stages of pathogenesis (298; 301). Although work in this thesis has demonstrated that *F. gigantica* may impact on the sensitivity of bTB diagnosis using the IFN-γ assay, future work should investigation the impact of the co-infection taking into account different parasite burdens and time points post-infection.

To maximise sensitivity of overall bTB testing, use of both the SCITT and IFN-γ assay potentially increased the sensitivity of detecting bTB positive cattle. As both tests identify different test positive populations (161). Although we cannot confirm that sampled cattle had bTB in this setting, due to the absence of a gold-standard diagnostic, cattle were identified as positive on one test and not the other hence there may be benefit in using the tests in combination or parallel testing to maximise sensitivity. The benefit of using IFN-γ assay in parallel in Cameroon would need to be further explored particularly in regard to increased costs. Also *F. hepatica* co-infections have been reported to reduce SCITT immune responses in *M. bovis* infected cattle (302; 299). Considering the impact of *F. gigantica* co-infection on IFN-γ responses demonstrated in this thesis, future work should investigate the impact of *F. gigantica* co-infection on both IFN-γ assay and SCITT results.

Pastoral cattle had similar levels of bTB reported in other extensive pastoral systems in SSA (101; 82; 442; 443), however few studies have used the IFN-γ assay to identify risk factors for bTB positivity in pastoral or dairy cattle in SSA. The differences in husbandry practices between pastoralist and dairy farmers, highlighted

in chapter 7, justified investigating these two populations in Cameroon separately. Factors associated with cattle being bTB positive, using the IFN-γ assay, were different in pastoral and dairy populations partly due to differences in population structure and husbandry practices.

Female pastoral cattle had lower odds of being identified as IFN-γ positive (OR: 0.38 CI: 0.21-0.70), that might be related to females having an increased odds of being classed as a false negative due to their advanced age. Although, other studies in Cameroon have not found an association between sex and bTB positivity using other diagnostics (114; 120; 116; 115), association with increased odds of being bTB positive in older females was also highlighted in chapter 4 and in the final MLR models as there was a positive interaction between age and females (tables 8.10 and 8.10; despite not being statistically significant inclusion did improve overall model fit). Female pastoral cattle are kept for longer than male cattle in Cameroon (Chapter 7 and (314)) and IFN-γ responses can become anergic in chronic *M. bovis* infections (164). As male cattle are kept for shorter periods (314) they may be in earlier stages of infection that are detected by the IFN-γ assay (48). Also females might be at less risk of bTB because they are exposed to environmental stressors, that could result in suppression of *M. bovis* immune responses, more frequently than male cattle. For example stress related to calving can cause generalised immunosuppression (374). Due to the their longevity within the herd compared to their male counterparts, females may be more prone to malnutrition (82) and chronic parasite infections (444) that result in generalised immunosuppression. Although not statistically significant, treating with a trypanocide was associated with an increased odds of being bTB positive. As trypanosomes interact with host immune responses, they may impact on *M. bovis* immune responses of the individual cattle. Trypanosome infections have also been noted with haematological changes that may impact on other co-infecting pathogens through immunosuppression (308; 445), highlighting the need to

understand co-infection dynamics beyond single pathogen interactions. Alternatively increased odds related to not using trypanocides, might be a proxy for poor husbandry practices that increase the risk of *M. bovis* transmission (82; 444).

Pastoral cattle in larger pastoral herds ($\geq$50+ cattle) were at greater odds of bTB. Increased contact between cattle in larger herds in intensive production systems, have been shown an increased risk of being bTB positive (446; 56; 447; 448). Pastoral herds in Tanzania, with similar husbandry practices to Cameroonian pastoral herds, were also reported to have increased risk of being bTB positive when $\geq$50+ cattle (35). Additionally defining a herd is difficult in Cameroon with multiple herds of cattle being owned by the same individual. Multiple herds may be owned by different family members however they can be merged at different times (314). Hence increased odds may be related to interactions between cattle between "herds", rather than necessarily herd size (449). Further exploration of pastoral cattle movements, in association with bTB risk, is warranted in Cameroon.

Environmental factors were shown to reduced the odds of pastoral cattle being bTB positive in Cameroon and might be a proxy for extensive management of pastoral cattle reducing bTB transmission. For example grazing with antelope also decreased the odds of transmission. Antelope species have been reported infected with *M. bovis* (107) and in some cases implicated as part of the transmission cycle in pats of SSA (413). Although susceptibility of Cameroonian antelope species has not been specifically investigated, decreased odds might be related to the variable being a proxy for extensive management of cattle. Extensive management practices may reduce the transmission pressure of *M. bovis* transmission compared to intensive cattle rearing systems (139). As most cattle in Cameroon are grazed on communal pastures (Chapter 7) contamination from *M. bovis* may be diluted by large grazing areas (442) and sunlight desiccation of *M. bovis* (62). Drinking from streams was also

found to decrease the odds of bTB positivity in cattle. Water troughs have been associated with *M. bovis* transmission due to the moist wet environment, favourable for *M. bovis* survival, and encouraging cattle to congregate (450). However drinking from streams may limit *M. bovis* transmission by allowing cattle more room to drink and minimising congregation around a single water source (57).

No association between sex, age and IFN-γ positivity in dairy cattle, as most dairy cattle were female and kept for <5 years (Chapter 7). Although not statistically significant, increased bTB positivity was associated with larger dairy herds. Herd size might be a proxy for increased contact between dairy cattle in a stalled housing system, with more cattle being kept in one single air space likely to increase *M. bovis* transmission (451). Similarly two Ethiopian studies noted severe bTB pathology (452) and more IFN-γ positive cases (178) in stalled housing systems compared to cattle kept at pasture. Also cattle in the Jakiri co-operative group were associated with a higher odds of IFN-γ positivity. From inception, Bamenda and Santa cooperatives sourced cattle from Kenyan herds (339). The Jakiri co-operative was set 7 years after Bamenda and Santa co-operatives and cattle were sourced from within Cameroon. Although their exact origin was unknown to farmers, it may be that these cattle may had been introduced from herds with established *M. bovis* infection.

In this study using the IFN-γ assay, has been demonstrated that bTB is endemic in Cameroonian cattle populations. Yet the impact of *F. gigantica* co-infection could substantially underestimate bTB prevalence estimates when using the assay, due to false negative test results. Prevalence of bTB is likely to be higher in the NWR compared to the VD. Differences in risk factors associated for IFN-γ positivity in pastoral and dairy cattle, highlights that *M. bovis* transmission may be related to differences in husbandry practices and bTB control may require different approaches in the two cattle populations.

Chapter 9

Discussion and conclusions

9.1 Thesis outcomes

As part of the larger CAMbTB project, the overall aim of this thesis was to investigate the epidemiology of bovine tuberculosis (bTB) in Cameroon, primarily using the IFN-γ assay, to highlight the significance of bTB to animal and human health. In order to accurately describe bTB epidemiology, five objectives were outlined:

- **Chapter 4**: What are the reasons for false positive and false negative test results when using the IFN-γ assay in Cameroon?
- **Chapter 5**: Does *F. gigantica* co-infection affect diagnosis of bovine tuberculosis in Cameroon?
- **Chapter 6**: Can a newly developed *F. gigantica* ELISA detect *F. gigantica* exposure in cattle?
- **Chapter 7**: What is the current level of awareness of bovine and zoonotic tuberculosis in cattle rearing communities?
- **Chapter 8**: What is the prevalence of bovine tuberculosis in different cattle rearing communities in Cameroon, comparing the IFN-γ assay to SCITT estimates?

Abattoir and cross-sectional studies were designed and conducted in the NWR and VD of Cameroon in 2011-13, sampling cattle from pastoral and dairy cattle populations, and the data used to address the aim of this thesis.

The results from the abattoir study were used to assess performance of the IFN-γ assay, relative to other bTB diagnostic tests. Although the IFN-γ assay had similar specificity (SP: 94.8-95.7%) as reported elsewhere (SP: 85-99.6% (78)), it was less sensitive (SE: 34.7-45.5%) than expected (SE: 73-100% (78)). Factors influencing false negative IFN-γ results were explored to be taken into account when describing

bTB epidemiology in Cameroonian cattle. False negative results were shown to be more likely in Fulani cattle and with evidence of *F. gigantica* pathology. Specifically *F. gigantica* co-infection with *M. bovis* was shown to reduce IFN-γ assay sensitivity by 20.3%, compared to *M. bovis* only infected cattle, and co-infected cattle were also more likely to have TB lesions present. Thus a *F. gigantica* antibody ELISA was developed (SE 85.0%; SP: 90.3%), to take into account *Fasciola* co-infection when using the IFN-γ assay in live cattle.

Secondly two cross-sectional studies were undertaken to describe the public health concerns of and epidemiology of bTB in pastoral and dairy cattle populations. Awareness of bTB in cattle was high in North West Region pastoralists (67.1%) and dairy farmers (73.9%) but lower in pastoralists from the Vina Division (40.8%). Disease awareness in Cameroon may be related to study site and gender. Awareness of zoonotic tuberculosis was low (2.0-21.7%) however resale of raw milk was commonplace, highlighting the potential public health risk from zTB. Pastoralists and dairy farmers were also shown to differ culturally and in their cattle husbandry practices, emphasising the need to investigate bTB epidemiology in these two populations separately.

Overall bTB appears to be endemic in Cameroon's pastoral and dairy cattle populations. The IFN-γ assay and the SCITT identified different cattle bTB positive, however reasons for disagreement were unable to be identified. A higher prevalence was noted in dairy cattle (25.0%, CI: 16.0-37.3%) compared to pastoral cattle in the NWR (13.2% CI: 11.0-15.8%) and VD (8.1%, CI: 6.3-10.3%) when using the IFN-γ assay and the SCITT in parallel. Differences in husbandry practices between pastoral and dairy cattle may account for differences in bTB prevalence within these two cattle populations. However the *F. gigantica* antibody ELISA was unequivocal in identifying the impact of *F. gigantica* co-infection on estimated bTB prevalence. Thus

further studies are required to identify the impact of *F. gigantica* co-infection on bTB diagnostics in Cameroon and beyond.

9.2 Implications, limitations and future research

The relationship between bovine tuberculosis status and interferon-gamma assay performance

In this thesis, the IFN-γ assay was used in the abattoir and cross-sectional studies to define bTB status of cattle. These investigations were useful in selecting a diagnostic cut-off ($\geq$0.1) for use of the assay in Cameroonian cattle and could be useful in other SSA countries, where the assay has been under utilised. Calculating accurate sensitivity and specificity of the IFN-γ assay allows the performance of the assay in Cameroon to be compared to its performance in other countries where the assay has been used.

Subsequently, identifying factors that could affect the assay's performance to define bTB status of cattle is important. These factors can be broadly split into two types, firstly "diagnostic test factors" that influence the performance of a diagnostic test to identify an animal's true bTB status (e.g. False positive and negative results) and secondly "true risk factors" that truly influence an animal's risk of being bTB positive (e.g. True positive and negative results). When investigating bTB epidemiology, identifying diagnostic test factors are just as important as identifying true risk factors as diagnostic test factors affect interpretation test results used to estimate bTB prevalence (summarised in figure 9.1).

Considering the low IFN-γ assay sensitivity (31.7-51.7%) estimated in this thesis, the assay was unlikely to have detected all *M. bovis* infected cattle. However the low

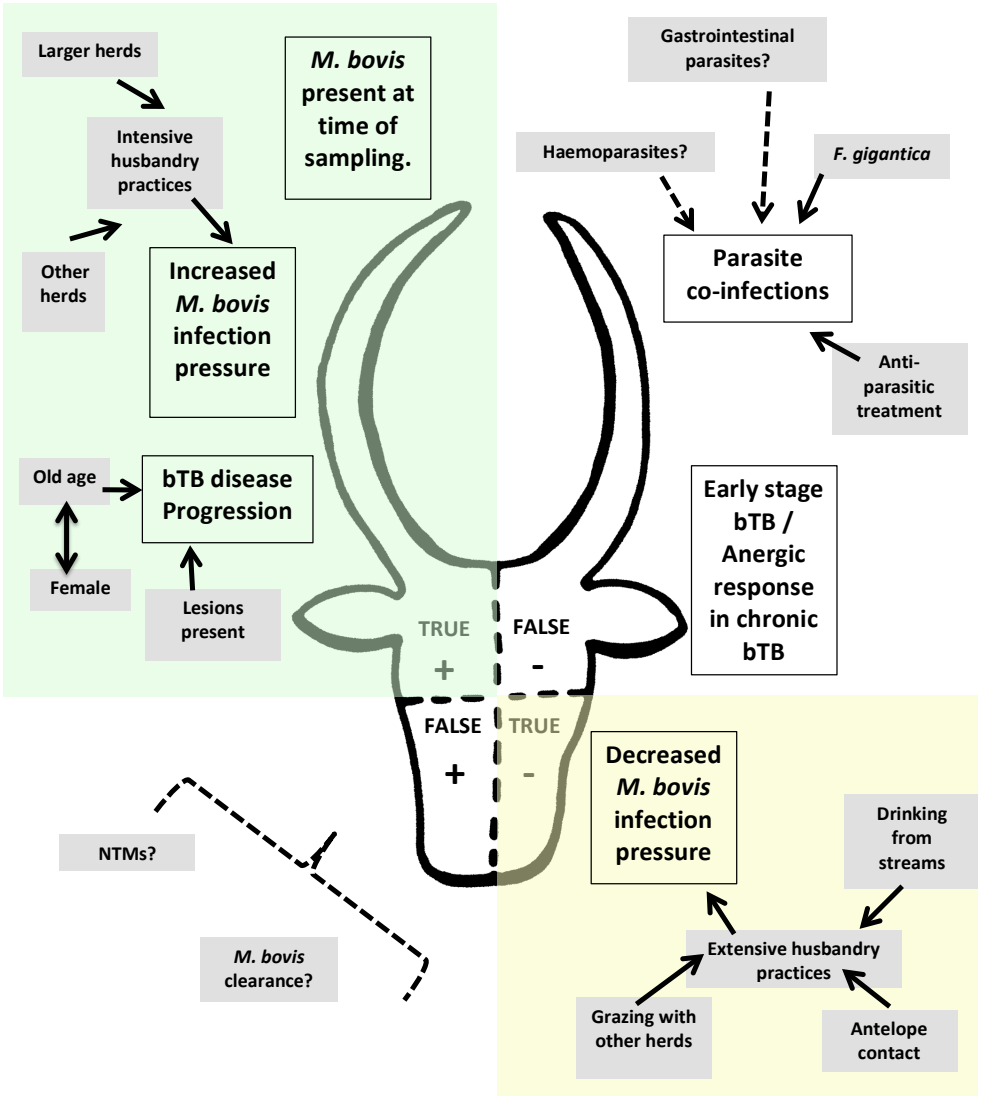


Figure 9.1: **Factors which influence interferon-gamma positive cattle.**
A schematic diagram to demonstrate the factors which potentially influence IFN-γ positivity in Cameroonian cattle. The green and yellow areas represent true IFN-γ positive and negative cattle respectively. Solid lines indicate factors explored in this thesis. Dotted lines indicate factors requiring further exploration.

sensitivity calculated was undertaken using a small subset of test responses and this may be unrepresentative of the true sensitivity of the assay within this population. In the absence of a gold standard diagnostic and using Bayesian statistical methods, non-gold standard latent class models can be used to estimate diagnostic sensitivity and specificity of the IFN-γ assay using data from large cross-sectional studies (376; 186; 441). By combining the abattoir and cross-sectional datasets, non-gold standard latent class models could be used to more accurately estimate the performance of the IFN-γ assay and the other bTB diagnostics in Cameroon. Whilst also considering logistical and practical aspects of the diagnostic tests, the results of the analysis could be useful in selecting the most suitable diagnostic test for different aspects of bTB surveillance in Cameroon.

Although *F. gigantica* co-infections and Fulani breed were identified as important factors contributing to false negative IFN-γ assay results, further work is required to identify factors that influence assay performance. Variation in immunes responses to *M. bovis* changes throughout the course of infection (46) and might affect interpretation of the IFN-γ assay (161). The design of the abattoir and cross-sectional studies limited investigations into how stage of bTB pathogenesis affects IFN-γ responses in individual cattle. Longitudinal studies in naturally infected populations, might be useful for investigating for how immune responses change over the lifetime of infected cattle. Additionally most published studies investigate immunological responses to *M. bovis* in *Bos taurus* cattle, yet immune responses in *Bos indicus* have been reported to differ from those in *Bos taurus* cattle (97; 35; 120). As both *B. indicus* and *B. taurus* cattle are present in Cameroon, future studies should specifically investigate differences in immune response to *M. bovis* as this might impact on diagnostic test interpretation.

Bovine tuberculosis, *Fasciola* and other parasitic co-infections

Uniquely this thesis ties together many aspects of the *M. bovis* and *F. gigantica* co-infection interaction at the individual animal level. *Fasciola gigantica* co-infection has a potential impact on the pathological development and diagnosis of bTB.

Fasciola gigantica co-infection was associated with presence of visible TB lesion development. Development of TB lesions is evidence of pathological progression with *M. bovis* infection. While lesions are evidence of the infected host mounting an immune response to *M. bovis*, development of TB lesions may lead to onward transmission of *M. bovis* (72). In Cameroon, development of lesions may be of benefit to public health and surveillance in abattoirs, however it is unclear what impact *F. gigantica* co-infection has on transmission of *M. bovis*. It has been shown that cattle co-infected with *F. hepatica* have reduced *M. bovis* burdens (301), yet in the abattoir study *M. bovis* burden was not recorded during the culture process. Further work on burden and shedding of *M. bovis* with *F. gigantica* co-infection would be useful to understand if co-infection affects transmission dynamics.

Fasciola gigantica co-infection was also associated with depressed IFN-γ responses and has implications for bTB diagnosis where the co-infection is present. As *F. gigantica* may lead to false negative IFN-γ results, bTB prevalence could be underestimated within Cameroon. Although the *F. gigantica* antibody ELISA did demonstrate that the exposure to *F. gigantica* was common in pastoral herds, emphasising that co-infection could impact bTB diagnosis across Cameroon, the ELISA was not useful in measuring the impact of *F. gignatica* co-infection on bTB diagnosis in live cattle. Probably because the *F. gigantica* antibody ELISA detected exposure rather than current infection. Thus further research should investigate the impact of *F. gigantica* co-infections on bTB diagnosis using *F. gigantica* diagnostics that assess current infection, such as FWEC or copro-antigen ELISAs.

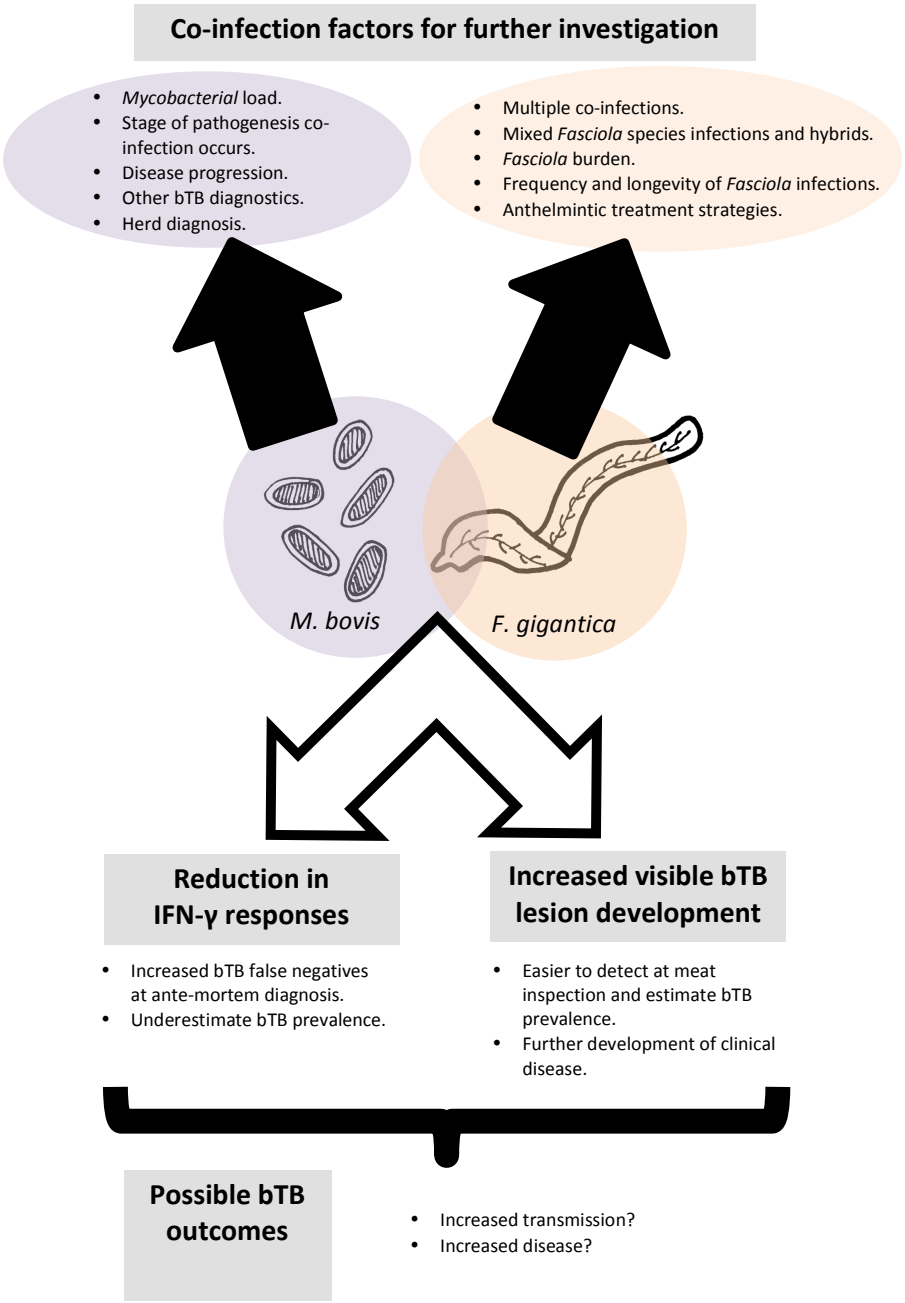


Figure 9.2: **Outcomes of *F. gigantica* co-infection on bTB diagnosis identified.** (White markers). With points for further investigation on *Fasciola* co-infection interactions with *M. bovis* (Black markers).

Reduction of the SCITT responses with *F. hepatica* co-infection have been reported and potential leading to false negative SCITT results (302). The affect of *F. gigantica* co-infection on the SCITT could not be investigated in this thesis, as it was not possible to keep cattle for 72 hours prior to slaughter in the abattoir study. Future studies that use the SCITT in Cameroonian cattle, should consider investigating the potential impact of *F. gigantica* co-infection on SCITT diagnosis to improve accuracy of bTB prevalence estimates.

However this thesis does not cover all aspects of the co-infection dynamic. Limitations are partly due to the design of the studies and logistics of sampling from a naturally infected population (summarised in figure 9.2). Multiple parasite factors are likely to affect the interaction between *M. bovis* and *F. gigantica*, such as parasite burden and length of infection (Figure 9.2), however these factors are yet to be explored with *F. gigantica* co-infections. For example studies on *M. bovis* and *F. hepatica* co-infection have demonstrated that effect on bTB diagnosis is determined by the order of infection of the two organisms (302). Furthermore we also do not know how bTB pathogenesis and diagnosis will be affected by multiple co-infections, such as trematodes, nematodes, haemoparasites in their numerous combinations. Certainly polyparasitism is common within ecosystems and multiple infections are likely to have different interactions and immunological consequences (388; 293; 453; 290). Conversely anti-parasitic treatments prior bTB testing might counteract the affect of parasitic co-infections on bTB diagnosis. Work by Ezenwa demonstrated buffalo not treated with anthelmintic boluses were more susceptible to bTB and had lower IFN-γ responses (454). Given the tropical climate in Cameroon, the potential impact of polyparasitism on bTB diagnosis requires further exploration before the presence of *F. gigantica* co-infection is used to adjust the interpretation of bTB diagnostics.

Ultimately co-infection work in this thesis, has implications for diagnosing bTB in Cameroon and beyond. In the future, mathematical modelling could be useful to calculate the probability of individual cattle being "bTB positive" by taking into account variables that explain the complex interaction between co-infections and *M. bovis*. Such models could be used in countries which wish to eradicate bTB by defining a probability of an animal being "bTB positive" rather than solely relying on a simple positive or negative result.

Bovine tuberculosis in Cameroon

Bovine tuberculosis appears to be endemic in cattle populations in Cameroon and is a potential zoonotic risk within cattle-rearing communities. Specifically, bTB prevalence in pastoral and dairy cattle populations appear to be similar to estimates in previous research studies (115; 77; 120) and comparable to similar production systems elsewhere in SSA (75; 35; 76; 57). Risk factors for bTB positivity appear to be specific to pastoral and dairy cattle populations. It is likely that bTB control measures need to be tailored to the different production systems (Figure 9.1). However prior to garnering support for implementation of additional control measures within cattle-rearing communities, there is a need for further understanding of the wider implications of bTB in Cameroon.

Although zoonotic transmission was not directly investigated in this thesis, an additional study associated within the larger CAMbTB project (Section 3.1) analysed sputum samples from patients presenting at local TB clinics around the NWR in 2013-14. Three of patients included in the study were infected with *M. bovis* (n=179), although it was unclear how these individuals were infected such as through consuming products infected with *M. bovis* or via aerosol transmission.

Prior instigating additional food safety measures to protect public health, the epidemiology of zTB in cattle-rearing communities should prioritised for future research. There was little awareness of bTB in cattle-keeping communities despite milk consumption and resale being high. It is unclear if the *M. bovis* is frequently secreted into milk of bTB positive cattle and if current methods used to heat or sour milk in Cameroon are already sufficient to destroy *Mycobacteria.* However if milk-borne transmission is identified as a significant problem within cattle-rearing communities, education programs could focus upon the risk posed from infected cattle and food preparation practices (455; 456). Research in this thesis has highlighted differences in milk processing practices and the cattle-rearing demographic in pastoral and dairy communities. Highlighting that if education programs were conducted in the future, content of the programs would need to be specifically tailored to suit the needs of each community.

Despite the limitations in test sensitivity, meat inspection in abattoirs is a simple cost-effective method of bTB surveillance and should be continued to protect public health. However, control of bTB in live cattle in Cameroon is more challenging. National test and slaughter programs have been useful in high income settings to improve control and elimination of bTB (41; 45), yet are unlikely to be useful in Cameroon. Test and slaughter programs require compensation schemes for slaughtered cattle and in a low income country, like Cameroon, they would be unaffordable. Strict biosecurity and registration of all cattle are also prerequisites for test and slaughter control programs. In the large pastoral cattle population in Cameroon, there is minimal biosecurity between herds and tracking cattle movements is absent. Furthermore although extensive husbandry practices appear to have lower risk of bTB in cattle, it is likely that intensification of cattle-rearing will continue into the future. Particularly in the NWR, where demand for dairy products is growing

rapidly, pastoral nomadic grazing practices are coming under increased conflict with crop farmers and promoting intensification of cattle rearing (42). Hence if bTB is to be controlled in Cameroon, novel approaches may need to be explored.

Vaccination against bTB has been advocated for use to minimise transmission and clinical disease in cattle in low-income countries (61; 31). Use of the BCG vaccine has been demonstrated to reduce pathological development (260; 132) and could be trialled in Cameroon to understand the effects on bTB transmission. If efficacious, BCG vaccination could be a viable option in the future as it could be included in annual vaccination programs of other cattle diseases, that are currently paid for by cattle keepers and MINEPIA. However extensive research is required prior vaccination being implemented on a large scale. For example a potential consequence of *Fasciola* co-infection is reduced response to BCG vaccine antigens(98) could impact on the efficacy of vaccination programs.

The Fulani *B. indicus* cattle breed was associated with bTB status in the thesis. Differences in susceptibility to bTB has been linked to the genotype of Holstein cattle (394; 395). Thus if genetic susceptibility to bTB was investigated further in Cameroonian cattle, in both *B. indicus* and *B. taurus* breeds, selective breeding programs may be useful in assisting with controlling bTB in the future.

In conclusion, this thesis highlights that the IFN-γ assay can be used to identify bTB positive cattle in Cameroon. However *F. gigantica* co-infection is likely to contribute to false negative diagnoses when using the IFN-γ assay and impact of co-infection should be taken into account when estimating of bTB prevalence. Ultimately with human and cattle contact ingrained in the culture of Cameroonian cattle keeping communities; improvements in public health protection against *M. bovis* would be a vital first step to maintain community health.

Appendices

Appendix A

Pastoral cross-sectional study population: Number of herds registered at ZVSCC in the North West Region and Vina Division in 2013.

North West Region			
Division	**ZVSCC name**	**ZVSCC code**	**Number of herds registered in 2013**
Boyo	Akeh	YAY	106
Boyo	Belo	YBO	71
Boyo	Fonfuka	YFA	11
Boyo	Fundong	YFG	138
Boyo	Konene	YKE	129
Boyo	Mungong	YMG	84
Boyo	Njinikom	YNM	10
Bui	Bamti	UBI	121
Bui	Ber	UBR	1
Bui	Dzeng	UDG	125
Bui	Elak Oku	UEU	16
Bui	Ibal Oku	UIU	39
Bui	Jakiri	UJI	49
Bui	Kumbo	UKO	190
Bui	Lip	ULP	67
Bui	Mbiame	UME	217
Bui	Mbokam	UMM	30
Bui	Mbonso	UMO	41
Bui	Sop	USP	58
Bui	Tadu	UTU	73
Bui	Tatum	UTM	107
Bui	Vikovi	UVI	73

Bui	Wainamah	UWH	93
Donga Mantung	Akweto	GAO	138
Donga Mantung	Binka	GBA	144
Donga Mantung	Gida Njikum	GGM	143
Donga Mantung	Kom	GKM	69
Donga Mantung	Misaje	GME	85
Donga Mantung	Nkambe	GNE	141
Donga Mantung	Ntim	GNM	77
Donga Mantung	Ntumbaw	GNW	214
Donga Mantung	Nwa	GNA	147
Donga Mantung	Sabongari	GSI	94
Donga Mantung	Yang	GYG	36
Menchum	Abomefang	KAG	20
Menchum	Bu	KBU	25
Menchum	Essimbi	KEI	10
Menchum	Esu	KEU	125
Menchum	Fura Awa	KKA	1
Menchum	Mmen	KMN	88
Menchum	Weh	KWH	52
Menchum	Wum	KWM	119
Menchum	Zhoa	KZA	21
Mezam	Akum	ZAM	53
Mezam	Awing	ZAG	98
Mezam	Bamendakwe	ZME	29
Mezam	Bafut	ZBT	4

Mezam	Baligam	ZBM	25
Mezam	Bambali	ZBI	17
Mezam	Bawock	ZBK	12
Mezam	Bossa	ZBA	21
Mezam	Finge	ZFE	12
Mezam	Kedjom Keku	ZKU	13
Mezam	Mankon	ZMN	15
Mezam	Mundum	ZMM	113
Mezam	Ntambeng	ZBG	21
Mezam	Pinyin	ZPN	127
Mezam	Sabga	ZSA	135
Mezam	Santa	ZST	40
Mezam	Tubah	ZTH	20
Momo	Acha Tugi	OAI	94
Momo	Ajei	OAJ	10
Momo	Andek	OAK	48
Momo	Ashong	OAG	40
Momo	Batibo	OBO	7
Momo	Enyoh	OEH	3
Momo	Etwii	OEI	13
Momo	Guzang	OGG	21
Momo	Mbengwi	OMI	37
Momo	Njikwa	ONA	22
Momo	Oshie	OOE	33
Momo	Teze	OTE	10

Momo	Tinechung	OTG	20
Momo	Widikum	OWN	16
Ngoketunjia	Babessi	NBI	27
Ngoketunjia	Babungo	NBO	98
Ngoketunjia	Bafanji	NBJ	1
Ngoketunjia	Bali Kumbat	NBT	74
Ngoketunjia	Bamalang	NBG	5
Ngoketunjia	Bangolan	NBN	29
Ngoketunjia	Ndop	NNP	92

Table A.1: **Pastoral cross-sectional study population: Number of herds registered at ZVSCC in the North West Region in 2013.**

Vina Division			
Subdivision	**ZVSCC name**	**ZVSCC code**	**Number of herds registered in 2013**
Belel	Bakari Bata	VBB	72
Belel	Beka Modibo	VMO	38
Belel	Belel	VBL	55
Belel	Djilougou	VDO	50
Belel	Idool	VIL	62
Belel	Tello	VTO	48
Belel	Tournigal	VTL	62
Martap	Beka Mangari	VBM	149
Martap	Likok	VLK	103
Martap	Lougga	VLA	132

Martap	Makor	VMR	21
Martap	Mandourou	VMU	46
Martap	Martap	VMP	119
Martap	Sebore Djangol	VSD	98
Mbe	Mbe	VME	29
Mbe	Sassa Mbari	VSM	16
Mbe	Wack	VWK	16
Ngan Ha	Lafia Didango	VNO	0
Ngan Ha	Mbang Foulbe	VMG	46
Ngan Ha	Mbang Mboum	VMB	26
Ngan Ha	Ngan Ha	VGH	110
Ngaoundere I	Lahore Vina	VLV	31
Ngaoundere II	Ngaoundere	VNE	25
Ngaoundere III	Dang	VNG	50
Ngaoundere III	Margol	VML	33
Nyambaka	Dibi	VDI	32
Nyambaka	Galdi	VGI	74
Nyambaka	Kognoli	VKI	91
Nyambaka	Mangom	VMM	98
Nyambaka	Nyambaka	VNA	29
Nyambaka	Wassande	VWM	136

Table A.2: **Pastoral cross-sectional study population: Number of herds registered at ZVSCC in the Vina Division in 2013.**

Appendix B

Dairy cross-sectional study population: Number of herds and cattle registered at the MINEPIA Regional Office in Bamenda, North West Region in 2013.

Cooperative group	Cooperative group code	Division	Sub-division	Total number of owners	Total number of Cattle
1. Bamenda	DHB	Mezam	Bamenda I	52	114
2. Santa	DHS	Mezam	Santa	95	183
3. Verkovi	DHV	Bui	Jakiri	43	86
4. Akum	DHA	Mezam	Santa	12	49
5. Mbot	DHM	Donga Mantung	Nkambe	12	22
6. Bali	DHI	Mezam	Santa	8	22
7. Finge	DHF	Mezam	Tubah	7	16
			Total	229	492
			Total sampled from	190	383

Table B.1: **Dairy cattle cross-sectional study population in the North West Region.**
Numbers of owners and cattle by cooperative group with totals displayed. Of the seven cooperative groups the three sampled, along with total number of owners and cattle, are highlighted in grey.

Appendix C

Abattoir animal form

Unique Animal Identifier_____ Animal N^{o}:

Cameroon bTB Abattoir Study: Cattle Examination Form

1. **General Information:**

Abattoir		**Date**	**/ /**
Butcher		**Sex**	**M F U**
Division of Origin		**Breed**	**WF RF MX GU EX OT UN**
Subdivision of Origin		**Colour**	**BLK BRN BKW BRW WHT WBK WBR UNK**
Village/ Town of Origin		**Age**	
		Dentition Score	**0 1 2 3 4 5 -999**

2. **Ante-mortem Examination:**

Body Condition Score	**-999 1 2 3 4 5**

Peripheral Blood Sample Type?	**Y or N**	
Whole Blood (Red Top)	**Y**	**N**
Heparinised Blood (Green Top)	**Y**	**N**

Additional Notes about the animal: _____

_____

_____

_____

Unique Animal Identifier_____ Animal N°:

3. Post-mortem Examination:

a. **TUBERCULOSIS LESIONS**

ID of tissue	Tissue Description	Lesion Present?	Pathological Score?	Scale of Lesion?	Size of Lesion?	Type of Lesion?	Sample Taken?
LYMPH NODES (LN)							
	Mandibular Left & Right	Y N					Y N
		Y N					Y N
	Medial Retropharyngeal	Y N					Y N
		Y N					Y N
	Mediastinal Crainal & Caudal	Y N					Y N
		Y N					Y N
	Bronchial Left and Right	Y N					Y N
		Y N					Y N
	Mesenteric	Y N					Y N
		Y N					Y N
	Supramammary	Y N					Y N
		Y N					Y N
	Prescapular	Y N					Y N
		Y N					Y N
	Prefemoral	Y N					Y N
		Y N					Y N
Total LN Lesion Score							
LUNG LOBES (LL)							
	Left Apical	Y N					Y N
	Left Cardiac	Y N					Y N
	Left Diaphargmatic	Y N					Y N
	Right Apical	Y N					Y N
	Right Cardiac	Y N					Y N
	Right Diaphragmatic	Y N					Y N
	Right Accessory	Y N					Y N
Total LL Lesion Score							
OTHER ORGANS							
	Pleura	Y N					Y N
	Uterus	Y N					Y N
	Udder	Y N					Y N
	Liver	Y N					Y N
	Kidney	Y N					Y N
	Skin	Y N					Y N
	Other:…….?	Y N					Y N

b. **FASCIOLOSIS LESIONS:**

Liver Pathology Score	**0**	**1**	**2**	**3**
Fasciola parasites identified in liver?	**Y**			**N**
Live (PBS) Fasciola parasite recovered?	**Y**			**N**
Dead (95% Ethanol) Fasciola parasite recovered?	**Y**			**N**

Appendix D

Cross-sectional questionnaire: English

University of Edinburgh Herd ID: __ __ __ / __ __ __

CAMbTB Cameroon Cattle Herder Field Study Questionnaire

Introduction to the Project:

Instructions for administering this questionnaire

1) Ask the questions as written. Remember the questionnaire is a measurement tool in the same way as a set of scales and consistency is critical. If prompting is necessary keep to a minimum and always use the same statement for each question. **Please ask all questions highlighted in grey unless not applicable; then refer to highlighted instructions to direct you to the next question to ask.**

2) Do not rush the interviewee, do not give any indication of your opinion, i.e. maintain a neutral expression, but show your interest.

3) Find somewhere private to conduct the interview away from any officials or neighbours who might affect the herdsman responses.

4) Remember **ALL ANSWERS ARE CONFIDENTIAL AS IS THE NAME OF THE INTERVIEWEE** and if another herdsman asks if his friend is included or about any of his responses you politely reply that you are unable to answer because we have promised everyone that the information is confidential. You need not say anything more.

5) In the event that a herdsman does not want to participate it is important at least to get the background details to allow us to classify the non-responders.

6) Before starting the questionnaire read the following statement.

"Thank you for agreeing to participate in this study of bovine tuberculosis, Liver Fluke/fasciolosis and other diseases in Cameroon. The project's purpose is to gain further understanding about various infectious diseases in Cameroonian cattle. The information you give will be used to understand why infections spread and help improve control of these diseases. Hopefully benefiting livelihoods by improving cattle health and production.

You have been selected by a random process from a list of names of people who had their herd vaccinated in the last 2 years. The choice of your herd in no way suggests there is anything wrong with your cows or other reasons. It is a choice just like the game of lottery. The names were put on the computer that then chose the names.

We now want to ask you some questions about how you manage **this particular herd you have here today**. This will help us assess various aspects of disease transmission and help in the interpretation of the results from the blood samples, and so give better advice to all herdsmen. All answers will be kept confidential and your answers will not be given to any other group. Your name will not be used in any report and only summary statistics will be quoted.

If you happy with this we would like to begin the questionnaire."

If verbal consent is granted then continue with the questionnaire.

Date: _____

University of Edinburgh Herd ID: __ __ __ / __ __ __

General Herd Information:

(0.0) GPS (*Circle*)	**Y**	**N**	**UNK**

(0.01) N			.					(0.02) E				.					(0.03) ALT	

(0.04) ZVSCC		(0.05) ZVSCC Code	

(0.06) Village		(0.07) Div		(0.08) SubDiv	

(0.09) Number of cattle presented		(0.10) Number of cattle sampled		(0.11) Interviewer	

(0.12) Additional comments	
If questionnaire stopped prior completion: reason why and question stopped at.	
(0.13) Question stopped at:	

1. Herdsman Information:

1.01 What is your full name?	

1.02 Who are you in regard to the cattle presented? (*Circle one*)				
Owner	**Herdsman**	**Caretaker**	**Other:** _____	**UNK**

1.03 How many years have you worked with cattle?	_____**Years**	**UNK**

1.04 What is your ethnic group?	

1.05 What is your education level? (*Circle one*)	**NONE**	**PRIMARY**	**SECONDARY**	**HIGHER**	**UNK**

1.06 How old are you?	_____**Years**	**UNK**

1.07 Record the owner's gender. (*Circle one*)	**M**	**F**

1.08 How many years have you kept cattle in this area?	_____**Years**	**UNK**

What other animals, other than cattle, do you currently keep/ rear at your homestead? (*Circle one per row*)			
(1.09) Sheep	**Y**	**N**	**UNK**
(1.10) Goats	**Y**	**N**	**UNK**
(1.11) Poultry	**Y**	**N**	**UNK**
(1.12) Cats	**Y**	**N**	**UNK**
(1.13) Dogs	**Y**	**N**	**UNK**
(1.14) Horses	**Y**	**N**	**UNK**
(1.15) Other	_____	**N**	**UNK**

2. Infectious Diseases:

This first section is about your perception of diseases you may have encountered and possibly have in your herd.

----------FOOT AND MOUTH DISEASE----------

2.01 Are you aware of a disease called "Foot and Mouth Disease"? (*Circle one*)	**Y**	**N**	**UNK**
If no or unknown, go to question 2.06			

2.02 What clinical signs do you associate with foot and mouth disease in cattle? *(Do not read the options and tick all as appropriate)*			
Weakness		Weight Loss	
Inappetence		Diarrhoea	
Coughing all the time		Breathing Difficulties	
Coughing intermittently		Bottle Jaw/ Neck Swelling	
Nasal Discharge		Lameness	
Recumbent		Enlarged Lymph Nodes	
Infertility		Aggression	
Mastitis/ Enlarged Udder		Reduced Milk Yield	
Eye Problems		Salivation/ Drooling	
Death		Arched Back	
Blood in Urine		Abortion	
Poor Coat		Separates from group	
Will not Breed		Swollen Testicles	
Other:		Does not know any clinical signs	

2.03 Have any of the cattle presented been sick from foot and mouth disease in the past 12 months? (*Circle one*)		
Y	**N**	**UNK**

2.04 Have any of your cattle died from foot and mouth disease in the past 12 months? (*Circle one*)		
Y	**N**	**UNK**
If no or unknown, go to question 2.06		

2.05 **If yes**, how many animals have died from foot and mouth disease in the past 12 months? (*Circle one*)					
0 cattle	**1-5 cattle**	**6-10 cattle**	**11-15 cattle**	**15+ cattle**	**UNK**

----------BOVINE TUBERCULOSIS----------

2.06 Are you aware of a disease called "Bovine Tuberculosis"? (*Circle one*)	**Y**	**N**	**UNK**
If no or unknown, go to question 2.18			

2.07 What clinical signs do you associate with bovine tuberculosis in cattle? *(Do not read the options and tick all as appropriate)*			
Weakness		Weight Loss	
Inappetence		Diarrhoea	
Coughing all the time		Breathing Difficulties	
Coughing intermittently		Bottle Jaw/ Neck Swelling	
Nasal Discharge		Lameness	
Recumbent		Enlarged Lymph Nodes	
Infertility		Aggression	
Mastitis/ Enlarged Udder		Reduced Milk Yield	
Eye Problems		Salivation/ Drooling	
Death		Arched Back	
Blood in Urine		Abortion	
Poor Coat		Separates from group	
Will not Breed		Swollen Testicles	
Other:		Does not know any clinical signs	

2.08 Have any of the cattle presented been sick from bovine tuberculosis, not including cattle that have died, in the past 12 months? (*Circle one*)		
Y	**N**	**UNK**

2.09 Have any of your cattle died from bovine tuberculosis in the past 12 months? (*Circle*)		
Y	**N**	**UNK**

2.10 How many animals have died from bovine tuberculosis in the past 12 months? (*Circle one*)					
0 cattle	**1-5 cattle**	**6-10 cattle**	**11-15 cattle**	**15+ cattle**	**UNK**

2.11 Have you been informed of any your cattle sold or slaughtered have bovine tuberculosis in the past 12 months? (*Circle one*)		
Y	**N**	**UNK**

2.12 Have you EVER been informed of any your cattle sold or slaughtered have bovine tuberculosis? (*Circle one*)		
Y	**N**	**UNK**

2.13 Have any of your cattle been tested for bovine tuberculosis? (*Circle one*)		
Y	**N**	**UNK**
If no or unknown, go to question 2.18		

2.14 **If yes,** how many months ago were they last tested for bovine tuberculosis?	_____**Months**

2.15 Were any bovine tuberculosis positive cattle reported on this test? (*Circle one*)		
Y	**N**	**UNK**
If no or unknown, go to question 2.18		

2.16 **If yes,** how many cattle tested positive to bovine tuberculosis? (*Circle one*)					
0 cattle	**1-5 cattle**	**6-10 cattle**	**11-15 cattle**	**15+ cattle**	**UNK**

2,17 What was done with test positive cattle? (*Free text*)	

----------FASCIOLOSIS----------

2.18 Are you aware of a disease called "Liver Fluke or Fasciolosis"? (*Circle one*)	**Y**	**N**	**UNK**
If no or unknown, go to question 2.25			

2.19 What clinical signs do you associate with Liver Fluke or Fasciolosis in cattle? *(Do not read the options and tick all as appropriate)*			
Weakness		Weight Loss	
Inappetence		Diarrhoea	
Coughing all the time		Breathing Difficulties	
Coughing intermittently		Bottle Jaw/ Neck Swelling	
Nasal Discharge		Lameness	
Recumbent		Enlarged Lymph Nodes	
Infertility		Aggression	
Mastitis/ Enlarged Udder		Reduced Milk Yield	
Eye Problems		Salivation/ Drooling	
Death		Arched Back	
Blood in Urine		Abortion	
Poor Coat		Separates from group	
Will not Breed		Swollen Testicles	
Other:		Does not know any clinical signs	

2.20 Have any of the cattle presented been sick from liver fluke infection in the past 12 months? (*Circle one*)		
Y	**N**	**UNK**

2.21 Have any of your cattle died from liver fluke infection in the past 12 months? (*Circle one*)		
Y	**N**	**UNK**

2.22 **If yes**, how many animals have died from liver fluke infection in the past 12 months? (*Circle one*)					
0 cattle	**1-5 cattle**	**6-10 cattle**	**11-15 cattle**	**15+ cattle**	**UNK**

2.23 Have you been informed of any your cattle sold or slaughtered have liver fluke infection in the past 12 months? (*Circle one*)		
Y	**N**	**UNK**

2.24 Have you EVER been informed of any your cattle sold or slaughtered have liver fluke infection? (*Circle one*)		
Y	**N**	**UNK**

2.25 Are you aware of any other major health concerns that have affected your cattle in the past 12 months? (*Free text*)

3. Routine Health Care:

The next set of questions asks you about if you provide veterinary care for your herd.

3.01 Have the cattle presented been vaccinated in the previous 12 months? (*Circle one*)		
Y	**N**	**UNK**

3.02 Have you treated the cattle presented with an anthelmintic/ wormer in the previous 12 months? (*Circle one*)		
Y	**N**	**UNK**
If no or unknown, go to question 3.06		

3.03 **If yes,** what was the name of the drug used? (*Free text*)

3.04 **If yes,** which cattle do you select to be treated? (*Circle multiple appropriate answers*)			
ALL	**SICK**	**NONE**	**UNK**

3.05 **If yes,** which age groups of cattle were treated? *(Circle multiple appropriate answers)*			
Calves (0-1 years)	**Young Stock (1-3 years)**	**Adult (3+ years)**	**UNK**

3.06 Have you treated the cattle presented for trypanosomiasis in the previous 12 months? (*Circle one*)		
Y	**N**	**UNK**
If no or unknown, go to question 4.01		

3.07 **If yes,** what was the name of the drug used? (*Free text*)

3.08 **If yes,** which cattle did you select to be treated? (*Circle one*)			
ALL	**SICK**	**NONE**	**UNK**

3.09 If yes, which age groups of cattle were treated? *(Circle multiple appropriate answers)*			
Calves (0-1 years)	**Young Stock (1-3 years)**	**Adult (3+ years)**	**UNK**

4. Reproduction

The next questions are about how you manage cattle breeding in your herd.

4.01 Have you used natural breeding in the previous 12 months? (*Circle one*)		
Y	**N**	**UNK**
If no or unknown, go to question 4.03		

4.02 What breed of bull is used for natural breeding? (*Free text*)

4.03 Have you used Artificial Insemination (AI) in the previous 12 months? (*Circle one*)		
Y	**N**	**UNK**
If no or unknown, go to question 5.01		

4.04 What breed of bull is used for Artificial Insemination (AI)? (*Free text*)

5. Grazing and Housing:

The next sets of questions are about how you manage grazing and the nutrition of these animals presented. This section does NOT regard transhumance as this will be discussed in a later section.

5.01 Have the cattle presented been allowed to graze in open pasture the in previous 12 months? (*Circle one*)		
Y	**N**	**UNK**
If no or unknown, go to question 5.09		

5.02 In the area regularly grazed by these cattle presented, is it (*Circle one*):		
Natural	**Improved**	**UNK**
If unknown, go to question 5.09		

5.03 Are any parts of the pasture grazed by these cattle presented, in the past 12 months, flooded or swampy? (*Circle one*)		
Y	**N**	**UNK**

5.04 How many other herds graze the same pasture, as these cattle, on a daily basis? (*Circle one*)					
0 herds	**1-5 herds**	**6-10 herds**	**11-15 herds**	**15+ herds**	**UNK**

Do any of these cattle presented come in contact with the following wild animals whilst grazing? (*Circle one per row*)			
(5.05) Buffalo	**Y**	**N**	**UNK**
(5.06) Antelope or deer	**Y**	**N**	**UNK**
(5.07) Warthog	**Y**	**N**	**UNK**
(6.08) Other	_____	**N**	**UNK**

5.09 Have the presented cattle been kept housed the majority of their time in the past 12 months?(*Circle one*)		
Y	**N**	**UNK**

5.10 Do you keep these cattle, presented, in a fenced enclosure over night? (*Circle one*)		
Y	**N**	**UNK**
If no or unknown, go to question 5.13		

5.11 **If yes,** do the cattle presented share the fenced enclosure with other herds? (*Circle one*)		
Y	**N**	**UNK**

5.12 How many other herds do your cattle share the fenced enclosure with? (*Circle one*)					
0 herds	**1-5 herds**	**6-10 herds**	**11-15 herds**	**15+ herds**	**UNK**

5.13 Have you fed these cattle presented any other supplements in the previous 12 months? (*Circle one*)		
Y	**N**	**UNK**
If no or unknown, go to question 5.15		

5.14 **If yes,** what did you feed? (*Free text*)

Which of the following do the presented cattle drink from on a regular basis ?: (*Circle one per row*)			
(5.15) Water troughs	**Y**	**N**	**UNK**
(5.16) Water canals	**Y**	**N**	**UNK**
(5.17) Streams	**Y**	**N**	**UNK**
(5.18) Lakes or Ponds	**Y**	**N**	**UNK**

5.19 How many other herds do your cattle contact at these watering points regulary? (*Circle one*)					
0 herds	**1-5 herds**	**6-10 herds**	**11-15 herds**	**15+ herds**	**UNK**

5.20 How many other herds use the same watering points as your cattle? (*Circle one*)					
0 herds	**1-5 herds**	**6-10 herds**	**11-15 herds**	**15+ herds**	**UNK**

6. Transhumance:

The next series of questions relate only when your herd go on transhumance, if this is applicable.

6.01 Did any of the cattle presented go on transhumance in the past 12 months? (*Circle one*)		
Y	**N**	**UNK**
If no or unknown, go to question 7.01		

6.02 What is the name of the area your cattle go to on transhumance?					
Area		**Div**		**SubDiv**	
UNK					

6.03 How many days walk from where the cattle are now is?	
_____Days	**UNK**

6.04 Have the cattle presented; grazed on flooded or swampy pasture whilst on transhumance in the past 12 months? (*Circle one*)		
Y	**N**	**UNK**

6.05 How many herds on average does your herd contact on a daily basis in the transhumance area? (*Circle one*)					
0 herds	**1-5 herds**	**6-10 herds**	**11-15 herds**	**15+ herds**	**UNK**

Do any of these cattle presented come in contact with the following wild animals whilst on transhumance? (*Circle one per row*)			
(6.06) Buffalo	**Y**	**N**	**UNK**
(6.07) Antelope or deer	**Y**	**N**	**UNK**
(6.08) Warthog	**Y**	**N**	**UNK**
(6.09) Other	_____	**N**	**UNK**

6.10 Which month did you go on transhumance? (*Circle as appropriate*)												
Jan	**Feb**	**Mar**	**Apr**	**May**	**Jun**	**Jul**	**Aug**	**Sep**	**Oct**	**Nov**	**Dec**	**UNK**

6.11 Which month did you return from transhumance? (*Circle as appropriate*)												
Jan	**Feb**	**Mar**	**Apr**	**May**	**Jun**	**Jul**	**Aug**	**Sep**	**Oct**	**Nov**	**Dec**	**UNK**

7. Cattle Sales:

The next series of questions are about purchase and sale of cattle.

7.01 Have you sold any cattle in the past 12 months? (*Circle one*)		
Y	**N**	**UNK**
If no or unknown, go to question 7.03		

7.02 How many times have you sold cattle in the past 12 months? (*Circle one*)

7.03 Have you bought any cattle in the past 12 months? (*Circle one*)		
Y	**N**	**UNK**
If no or unknown, go to question 7.05. If no or unknown, to both 7.01 and 7.03, go to question 8.01		

7.04 How many times have you purchased cattle in the past 12 months? (*Free text*)

7.05 Have sales or purchases been conducted at these places or with the following people in the past 12 months (*Circle multiple appropriate answers*)						
Markets	**"Buyem and sellems"**	**Breeder**	**Neighbour**	**Other Herder**	**OTHER** _____	**UNK**

8. Milk and Dairy Habits:

The final set of questions are about milking your cows and drinking their milk.

8.01 Do you or any of your family drink milk from these animals presented? (*Circle one*)		
Y	**N**	**UNK**
If no or unknown, go to question 8.07		

8.02 Do you treat or heat this milk drunk by your family? (*Circle one*)		
Y	**N**	**UNK**
If no or unknown, go to question 8.07		

8.03 Do you treat this milk by souring? (*Circle one*)		
Y	**N**	**UNK**
If no or unknown, go to question 8.05		

8.04 **If yes,** how long is milk soured for?	_____**days**	**UNK**
If no or unknown, go to question 8.05		

8.05 Do you treat this milk by heating? (*Circle one*)		
Y	**N**	**UNK**
If no or unknown, go to question 8.07		

8.06 **If yes,** how long is milk heated for?	_____**minutes**	**UNK**

8.07 Is the milk from these animals presented sold or given to other people other than your family? (*Circle one*)		
Y	**N**	**UNK**
If no or unknown, go to question 8.13		

8.08 Do you treat or heat the milk sold or given to other people? (*Circle one*)		
Y	**N**	**UNK**
If no or unknown, go to question 8.13		

9.09 Do you treat this milk by souring? (*Circle one*)		
Y	**N**	**UNK**
If no or unknown, go to question 8.11		

8.10 **If yes,** how long is this milk soured for?	_____**days**	**UNK**
If no or unknown, go to question 8.11		

8.11 Do you treat this milk by heating? (*Circle one*)		
Y	**N**	**UNK**
If no or unknown, go to question 8.13		

8.12 **If yes,** how long is this milk heated for?	_____**Minutes**	**UNK**

8.13 Is any of your milk mixed with milk from other herds prior treatment or heating? (*Circle one*)			
Y	**N**	**UNK**	**NA**

8.14 Do you produce any of the following dairy products from your cow's milk? (*Circle multiple appropriate answers*)					
Cheese	**Butter**	**Yoghurt**	**Other:** _____	**N**	**UNK**
If no or unknown, go to question 8.17					

8.15 **If yes,** do you or any of your family consume any of these dairy products? (*Circle one*)		
Y	**N**	**UNK**

8.16 **If yes,** do you sell or give any of these dairy products to other people other than your family?		
Y	**N**	**UNK**

8.17 Do you know of any diseases which people can get from drinking cow's milk? (*Circle one*)		
Y	**N**	**UNK**
If no or unknown, go to question 8.19		

8.18 What are the names of these diseases? (*Free text*)

8.19 Do you have any additional comments you wish to make about the questionnaire or the subjects covered?(*Free text, continue on other side if needed*)

Thank you very much for taking time to answer our questions, we will be in touch with our findings.

Appendix E

Cross-sectional questionnaire: Fulfulde

University of Edinburgh Herd ID: ___ ___ ___ / ___ ___ ___

CAMbTB Cameroon Cattle Herder Field Study Questionnaire: FULFULDE

Introduction to the Project:

Instructions for administering this questionnaire

1) Ask the questions as written. Remember the questionnaire is a measurement tool in the same way as a set of scales and consistency is critical. If prompting is necessary keep to a minimum and always use the same statement for each question. **Please ask all questions highlighted in grey unless not applicable; then refer to highlighted instructions to direct you to the next question to ask.**

2) Do not rush the interviewee, do not give any indication of your opinion, i.e. maintain a neutral expression, but show your interest.

3) Find somewhere private to conduct the interview away from any officials or neighbours who might affect the herdsman responses.

4) Remember **ALL ANSWERS ARE CONFIDENTIAL AS IS THE NAME OF THE INTERVIEWEE** and if another herdsman asks if his friend is included or about any of his responses you politely reply that you are unable to answer because we have promised everyone that the information is confidential. You need not say anything more.

5) In the event that a herdsman does not want to participate it is important at least to get the background details to allow us to classify the non-responders.

6) Before starting the questionnaire read the following statement.

"Mi yettima be jabugo hudugo be amin ko rarani nyaw jouru, balki be goddi nyawji. Kugal ngal dum wallitago anda hala nyawji fere-fere I naiji Cameroon. Ko mbiatámin wallitoto I anduki no nyawaji nai ngansirta heba no faddoridi. Dum nden boh wallitoto enjamu nai be nodi njehirta yerso doh bo jamaare fuh hebai boteh mai. Min kebi inde ma nderr tuphube nai nderr dubi didi salidi. Min ngaati inde himbe tuphube nai muenfuh nderr wakkati yel mai nderr Computer. Min bi ngasuptanami inde himbe de nga subi fuh. Nden nderr don inde ma wurti. Wáto I dum jordi bano njambo ko lotterie (guru-uh). Na wai gam nai ma I ngodi nyaw kobo hunde fere sam. To a jabi min jamete jamde korarani nai di di ngadduda be no njogoridadi. Dum wallai I andugo hala nyawuji nai no ngansirta be jawabuye kebadum I jirjam. Dum tammi wallitágo marbe nai sosai. Ko mbolududen fuh hádi halkundemen. Wala mo min mbiata nda ko mbida inde ma boh wangata dow dereji. Ko heba I jamare jamande tan hawrata nden wurtina dow dereji. Amma wala inde ngoddo kam wangata min ngettima massini."

If verbal consent is granted then continue with the questionnaire.

Date: _____

General Herd Information:

(0.0) GPS (*Circle*)	**Y**	**N**	**UNK**

(0.01) N			.					(0.02) E				.					(0.03) ALT	

(0.04) ZVSCC		(0.05) ZVSCC Code	

(0.06) Wuro		(0.07) Div		(0.08) SubDiv	

(0.09) Limgal nai gaddadi		(0.10) Limgal nai kuwanadi		(0.11) Jamowo	

(0.12) Additional comments	
If questionnaire stopped prior completion: reason why and question stopped at.	
(0.13) Question stopped at:	

1. Herdsman Information:

1.01 Noye indé ma fuh?	

1.02 An wóni moye I di nai gaddadi? (*Circle one*)				
Jómiji	**Gainákо**	**Califájo**	**Tana bédoh (goddo fare):** _____	**AND**

1.03 Dubi noye ko kududa be nai?	**Dúbe**_____	**AND**

1.04 A lenyol ngole?	

1.05 Jangirde ndeye marda? (*Circle one*)	**WALA**	**"PRIMARY"**	**"SECONDARY"**	**"HIGHER"**	**AND**

1.06 Dubi ma noye?	**Dúbi** _____	**AND**

1.07 Debbo na igorko (*Circle one*)	**G**	**D**

1.08 Dubi noye ndesirda nai I pellel ngeel?	**Dúbi** _____	**AND**

Dabbaji diye kowiti nai ndesuda I pellel ma? (*Circle one per row*)			
(1.09) Baali	**E**	**A**	**AND**
(1.10) Bei	**E**	**A**	**AND**
(1.11) Gerode	**E**	**A**	**AND**
(1.12) Musuji	**E**	**A**	**AND**
(1.13) Bosaji	**E**	**A**	**AND**
(1.14) Putchi	**E**	**A**	**AND**
(1.15) Tana maji (Goddi fere)	_____	**A**	**AND**

2. Infectious Diseases:

Aramde nde dum ko rarani andagal ma nyawuji be goddi nderr nai ma

----------FOOT AND MOUTH DISEASE----------

2.01 A andi hala nyaw bi adum mboru nah?(*Circle one*)	**E**	**A**	**AND**
If no or unknown, go to question 2.06			

2.02 Dume ngiata I nagge andinojummah I nge mari nyaw mboru? *(Do not read the options and tick all as appropriate)*			
Soinde sembe		Láfendam	
Nyamata		Sarol	
Dojjai ko ndeye		Sadirma foftugoh	
Dojjai nde go go		Wolo búta/ Dande búta	
Nyible I ila		Laire	
Wofágo		Ngenóje mawna	
Rimata		Hawre	
Felewre/ Yeire búta		Kossam famdita	
Gide nawóje		Jódai	
Mbáta		Bawo turingo	
Silla jijam		Wuftere	
Lebre nyidunde		Yidá njairi; sadawre	
Hojata		Bokoloje búta	
Tana máji (Goddum fere):		Wala ko andi I majum	

2.03 Wódi nderr nai di nyawdi mboru nderr lebbi sappo I didi salidinah? (*Circle one*)		
E	**A**	**AND**

2.04 Wódi nai mboru mbari nderr lebbi sappo I didi salidinah? (*Circle one*)		
E	**A**	**AND**
If no or unknown, go to question 2.06		

2.05 Nai noye mboru mbari nderr lebbi sappo I didi salidi? (*Circle one*)					
0 Nai	**1-5 Nai**	**6-10 Nai**	**11-15 Nai**	**15+ Nai**	**AND**

----------BOVINE TUBERCULOSIS----------

2.06 A andi ha nyaw jouru (soharu) nah? (*Circle one*)	**E**	**A**	**AND**
If no or unknown, go to question 2.18			

2.07 Dumé ngi ata I nagge andinojumma I nge mari nyaw jouru (soharu)? *(Do not read the options and tick all as appropriate)*			
Soinde sembe		Láfendam	
Nyamata		Sarol	
Dojjai ko ndeye		Sadirma foftugoh	
Dojjai nde go go		Wolo búta/ Dande búta	
Nyible I ila		Laire	
Wofágo		Ngenóje mawna	
Rimata		Hawre	
Felewre/ Yeire búta		Kossam famdita	
Gide nawóje		Jódai	
Mbáta		Bawo turingo	
Silla jijam		Wuftere	
Lebre nyidunde		Yidá njairi; sadawre	
Hojata		Bokoloje búta	
Tana máji (Goddum fere):		Wala ko andi I majum	

2.08 Nderr nai di wódi nyaw di jouru nah banda batudi nderr lebbi sappo ididi sálidi? (*Circle one*)		
E	**A**	**AND**

2.09 Nderr nai ma wódi din yaw jouru (saharu) mbari mderr lebbi sappo I didi salidinah? (*Circle*)		
E	**A**	**AND**

2.10 Nai noye jouru mbari nderr lebbi sappo I didi salidi? (*Circle one*)					
0 Nai	**1-5 Nai**	**6-10 Nai**	**11-15 Nai**	**15+ Nai**	**AND**

2.11 Wódi andindoma nderr nai ma soradi ko kirsadi nderr lebbi sappo I didi salidi wódi di be tawri nyaw jouru nah? (*Circle one*)		
E	**A**	**AND**

2.12 A medi andinego nderr nai ma soradi ko kirsaw I mari nyaw jouru nah? (*Circle one*)		
E	**A**	**AND**

2.13 Be médi rarugo nyaw jouru I nai ma nahi? (*Circle one*)		
E	**A**	**AND**
If no or unknown, go to question 2.18		

2.14 Lebbi noye hande diga be ndari nyaw jouru man?	**Lebbi _____**

2.15 Wódi nden nai be mbima I mari nyaw jouru nah? (*Circle one*)		
E	**A**	**AND**
If no or unknown, go to question 2.18		

2.16 To eeh; nai noye be tawi I mari nyaw jouru? (*Circle one*)					
0 Nai	**1-5 Nai**	**6-10 Nai**	**11-15 Nai**	**15+ Nai**	**AND**

2,17 No mardi nyaw man ngadanah? (*Free text*)	

----------FASCIOLOSIS----------

2.18 A andi hála nyaw balki nah? (*Circle one*)	**E**	**A**	**UNK**
If no or unknown, go to question 2.25			

2.19 Dume ngi ata I ngge andinojummah I nge mari nyaw balki? *(Do not read the options and tick all as appropriate)*			
Soinde sembe		Láfendam	
Nyamata		Sarol	
Dojjai ko ndeye		Sadirma foftugoh	
Dojjai nde go go		Wolo búta/ Dande búta	
Nyible I ila		Laire	
Wofágo		Ngenóje mawna	
Rimata		Hawre	
Felewre/ Yeire búta		Kossam famdita	
Gide nawóje		Jódai	
Mbáta		Bawo turingo	
Silla jijam		Wuftere	
Lebre nyidunde		Yidá njairi; sadawre	
Hojata		Bokoloje búta	
Tana máji (Goddum fere):		Wala ko andi I majum	

2.20 Mderr nai di wódi nyawdi nyaw balki nderr lebbi sappo I didi salidinah? (*Circle one*)		
E	**A**	**AND**

2.21 Wódi nai nyaw balki mbari nderr lebbi sappo I didi salidinah? (*Circle one*)		
E	**A**	**AND**

2.22 Nai noye nyaw balki mbari nderr lebbi sappo I didi salidi? (*Circle one*)					
0 Nai	**1-5 Nai**	**6-10 Nai**	**11-15 Nai**	**15+ Nai**	**AND**

2.23 Wódi andindoma nderr ná ma soradi ko kirsadi nderr lebbi sappo I didi salidi mardi nyaw balki nah? (*Circle one*)		
E	**A**	**AND**

2.24 A medi andinego nderr nai ma sorádi ko kirsadi wóda mardi nyaw balki nah? (*Circle one*)		
E	**A**	**AND**

2.25 Wódi nyawuji goddi ndámi nai ma nderr lebbi sapp i didi salidinah? (*Free text*)

3. Routine Health Care:

Jamde tockudde rarani no nyawndirta nai ma ndyefuh

3.01 Nai di kam tafama (tiphail) nderr lebbi sappo i didi sálidinah? (*Circle one*)		
E	**A**	**AND**

3.02 A hoeki nai di leeki bole nderr lebbi sappo i didi salidinah? (*Circle one*)		
E	**A**	**AND**
If no or unknown, go to question 3.06		

3.03 No inde lecki man? (*Free text*)

3.04 Dum diy nai suptuda nywnduda bóle? (*Circle multiple appropriate answers*)			
Fuh	**Nyawdi**	**Wâla**	**AND**

3.05 Nai wi diye be diye nyawnduda?			
Bickon (dúbi 0-1)	**Pamari (dúbi 1-3)**	**Mawadi (dúbi 3+)**	**AND**

3.06 Nyawndi nai ma lecki pial nderr lebbi sappo i didi salidinah? (*Circle one*)		
E	**A**	**AND**
If no or unknown, go to question 4.01		

3.07 No inde lecki man? (*Free text*)

3.08 Dum diye suptuda nywnduda bóle? (*Circle one*)			
Fuh	**Nyawdi**	**Wâla**	**AND**

3.09 Nai wi diye be diye nyawnduda? *(Circle multiple appropriate answers)*			
Bickon (dúbi 0-1)	**Pamari (dúbi 1-3)**	**Mawadi (dúbi 3+)**	**AND**

4. Reproduction

Jamde tockude rarani no nai ma ndimirta

4.01 Kaleldi katinirda I wamnugo nai ma nderr lebbi i didi nah? (*Circle one*)		
E	**A**	**AND**
If no or unknown, go to question 4.03		

4.02 Awre kalaldi ndeye kurtinirda? (*Free text*)

4.03 A hutiniri bate I wamnuka nai ma nah?? (*Circle one*)		
E	**A**	**AND**
If no or unknown, go to question 5.01		

4.04 Awre kalaldi ndeye kutinirda i bamnol bate? (*Free text*)

5. Grazing and Housing:

Jamde tockude rarani durngol be no nyamnirta nai ma koluti dabbol gam hanjuma I dum wara.

5.01 Nai di kam I ladde non nduri nderr lebbi sappo I didi salidi nah? (*Circle one*)		
E	**A**	**AND**
If no or unknown, go to question 5.09		

5.02 Don to nai di ndurata kam dum géne (*Circle one*):		
Ladde	**Géne Awáde**	**AND**
If unknown, go to question 5.09		

5.03 Ha di nduri nderr lebbi sappo i didi salidi wodi babe godde mdiam mabbi kóbó mari serbore nah? (*Circle one*)		
E	**A**	**AND**

5.04 Take goddi noye ndura tu I nockure man nderr lebbi sappo I didi? (*Circle one*)					
0 Tockere	**1-5 Tocke**	**6-10 Tocke**	**11-15 Tocke**	**15+ Tocke**	**AND**

Nderr di nai wódi pototiroji be dabbaji ladde di nah? (*Circle one per row*)			
(5.05) Mbana	**E**	**A**	**AND**
(5.06) Njama sirga were ladde	**E**	**A**	**AND**
(5.07) Gaduru ladde	**E**	**A**	**AND**
(6.08) Goddi fere	_____	**A**	**AND**

5.09 Nai di kam burunafu nderr súdu dim balata nderr lebbi sappo i didi salidinah?(*Circle one*)		
E	**A**	**AND**

5.10 Nai di kam nderr kowagol di mbalatanah? (*Circle one*)		
E	**A**	**AND**
If no or unknown, go to question 5.13		

5.11 Nai di kam I mbalda be nai goddi nah? (*Circle one*)		
E	**A**	**AND**

5.12 Tocke nai noye mbaldata be nai madi? (*Circle one*)					
0 Tockere	**1-5 Tocke**	**6-10 Tocke**	**11-15 Tocke**	**15+ Tocke**	**AND**

5.13 Anyamni nai di nyamdu goddum nderr lebbi sappo i didi salidi nah? (*Circle one*)		
E	**A**	**AND**
If no or unknown, go to question 5.15		

5.14 Ko nyamnudadi? (*Free text*)

Burnáfu I dume nai di njarata ?: (*Circle one per row*)			
(5.15) Kombóje ndiam	**E**	**A**	**AND**
(5.16) Iladi ndiam	**E**	**A**	**AND**
(5.17) Máje	**E**	**A**	**AND**
(5.18) Béli ko bulli	**E**	**A**	**AND**

5.19 Tocke nai noye burunafu nai ma di pottata to di njarata ndiam? (*Circle one*)					
0 Tockere	**1-5 Tocke**	**6-10 Tocke**	**11-15 Tocke**	**15+ Tocke**	**AND**

5.20 Tocke nai noye njarata I to nai ma njarata? (*Circle one*)					
0 Tockere	**1-5 Tocke**	**6-10 Tocke**	**11-15 Tocke**	**15+ Tocke**	**AND**

6. Transhumance:

Jamde tokudde de de ranrani to nai ma I sedoya.

6.01 Nai di kam wódi sedoidi nderr lebbi sappo i didi salinah? (*Circle one*)		
E	**A**	**AND**
If no or unknown, go to question 7.01		

6.02 No inde nockure nde nai ma sedoita?					
Nokume		**Div**		**SubDiv**	
AND					

6.03 Balde noye di kósata dig a do yágo sedirde máji?	
Balde_____	**AND**

6.04 i sedirde mai nai mai ndidri I nockuje ndiam dai ko seboje nderr lebbi sappo i didi salidinah? (*Circle one*)		
E	**A**	**AND**

6.05 Tocke noye nai ma kawrata nderr nyalwmare; I sedirdemaji? (*Circle one*)					
0 Tockere	**1-5 Tocke**	**6-10 Tocke**	**11-15 Tocke**	**15+ Tocke**	**AND**

i wódi nderr nai di kawroji be dabbaji ladde di nah? (*Circle one per row*)			
(6.06) Mbana	**E**	**A**	**AND**
(6.07) Mbaroga were ladde	**E**	**A**	**AND**
(6.08) Gaduru ladde	**E**	**A**	**AND**
(6.09) Goddi fere	_____	**A**	**AND**

6.10 Lewru nduye ndilluda dabbol? (*Circle as appropriate*)												
Jan	**Feb**	**Mar**	**Apr**	**May**	**Jun**	**Jul**	**Aug**	**Sep**	**Oct**	**Nov**	**Dec**	**AND**

6.11 Lewru nduye ngartuda dabbol? (*Circle as appropriate*)												
Jan	**Feb**	**Mar**	**Apr**	**May**	**Jun**	**Jul**	**Aug**	**Sep**	**Oct**	**Nov**	**Dec**	**AND**

7. Cattle Sales:

Jamde tockude rarani sougo i sorugo nai.

7.01 Wóda na sonuda nderr lebbi sappo i didi salidinah? (*Circle one*)		
E	**A**	**AND**
If no or unknown, go to question 7.03		

7.02 Nde nouye soruda nai nderr lebbi sappo i didi salidi? (*Circle one*)

7.03 Wóda nai soduda nderr lebbi sappo i didi salidi nah? (*Circle one*)		
E	**A**	**AND**
If no or unknown, go to question 7.05. If no or unknown, to both 7.01 and 7.03, go to question 8.01		

7.04 Nde noye soduda nai nderr lebbi sappo i didi salidi? (*Free text*)

7.05 Sodugo be sorugo i rarani be nah? (*Circle multiple appropriate answers*)						
Lúbe	**Kamkamba ko Saikaina**	**Bamnowo**	**Keddidirawo**	**Durowo**	**Goddum** _____	**AND**

8. Milk and Dairy Habits:

Jamde sakitide deh rarani birugo e yargo kossam.

8.01 An be himbe ma i njara kossam na di nah? (*Circle one*)		
E	**A**	**AND**
If no or unknown, go to question 8.07		

8.02 Kossam dam njaraton man on ngadanadam lecki kobo ndolla dam nah? (*Circle one*)		
E	**A**	**AND**
If no or unknown, go to question 8.07		

8.03 On dannina kossam dam nah? (*Circle one*)		
E	**A**	**AND**
If no or unknown, go to question 8.05		

8.04 Balde noye dam danninirte?	**Balde_____**	**AND**
If no or unknown, go to question 8.05		

8.05 On ndolla kossam dam nah? (*Circle one*)		
E	**A**	**AND**
If no or unknown, go to question 8.07		

8.06 Minti noye dam dollirte?	**Minti _____**	**AND**

8.07 On sora ko haeka himbe wobe kossam má nah? (*Circle one*)		
E	**A**	**AND**
If no or unknown, go to question 8.13		

8.08 On ngadanadam lecki ko ndolladam ko soron ko kockon himbe nah? (*Circle one*)		
E	**A**	**AND**
If no or unknown, go to question 8.13		

9.09 On danninadam nah? (*Circle one*)		
E	**A**	**AND**
If no or unknown, go to question 8.11		

8.10 Balde noye dam danninirte?	**Balde_____**	**AND**
If no or unknown, go to question 8.11		

8.11 On ndolla dam nah? (*Circle one*)		
E	**A**	**AND**
If no or unknown, go to question 8.13		

8.12 Minti noye?	**Minti_____**	**AND**

8.13 A hawra dam be kossam I ngoddam da ngadaradam lecki ko ndolladam nah ? (*Circle one*)			
E	**A**	**AND**	**Wadatako**

8.14 A wadira kossam dam karji godde nah? (*Circle multiple appropriate answers*)					
"Cheese"	**Nebbam**	**Pendidam**	**Goddum: _____**	**Wala**	**AND**
If no or unknown, go to question 8.17					

8.15 An ko himbe ma wobbe I nyama limtadum du nah? (*Circle one*)		
E	**A**	**AND**

8.16 Koluti himbe ma wobbe I nyama limtatum du nah?		
E	**A**	**AND**

8.17 A andi nyawuju di himbe póti nangagu to i njara kossam nah? (*Circle one*)		
E	**A**	**AND**
If no or unknown, go to question 8.19		

8.18 No inde nyawuji din? (*Free text*)

8.19 A wódi ko potuda beidugo I jamdede nah kobo ko rarani kúgal nga al nah?(*Free text, continue on other side if needed*)

Mi yeti ma i horsugo wackati ma I notago jamde amin. En matotirai i jawabuye amin.

Appendix F

Cross-sectional animal form

University of Edinburgh

Herd ID: ____/____

	ROB				HERDER									ROB					
ANIMAL ID	Gender	Breed	BCS	Dentition	Age(M/Y?)	Weight Loss?	Coughing	Diarrhoea	Not Eating	Breathing Difficulties	Worming (12 M)	Mastitis (FEMALE)	Abortion (FEMALE)	Comments	CST NECK SITE	PPD SITE	Day0 skin(mm)	Day3 skin(mm)	Samples taken?
01	M					Y	Y	Y	Y	Y	Y	Y	Y		L	A			Red
	F					N	N	N	N	N	N	N	N		R	B			Grn
02	M					Y	Y	Y	Y	Y	Y	Y	Y		L	A			Red
	F					N	N	N	N	N	N	N	N		R	B			Grn
03	M					Y	Y	Y	Y	Y	Y	Y	Y		L	A			Red
	F					N	N	N	N	N	N	N	N		R	B			Grn
04	M					Y	Y	Y	Y	Y	Y	Y	Y		L	A			Red
	F					N	N	N	N	N	N	N	N		R	B			Grn
05	M					Y	Y	Y	Y	Y	Y	Y	Y		L	A			Red
	F					N	N	N	N	N	N	N	N		R	B			Grn

CATTLE BREED CODES:

WF= White Fulani **GU= Gudali**
RF= Red Fulani **EX= Exotic**
MX= Mixed Breed **OT= Other**
UN= Unknown

TALLY OF CATTLE AGE GROUPS (5 REQ/ SAMPLE:

CALVES (0 P INCISORS): _____

YOUNGSTOCK (1-2 P INC): _____

ADULTS (3 + P INC): _____

University of Edinburgh

Herd ID: ____/____

	ROB				HERDER									ROB					
ANIMAL ID	Gender	Breed	BCS	Dentition	Age(M/Y?)	Weight Loss?	Coughing	Diarrhoea	Not Eating	Breathing Difficulties	Worming (12 M)	Mastitis (FEMALE)	Abortion (FEMALE)	Comments	CST NECK SITE	PPD SITE	Day0 skin(mm)	Day3 skin(mm)	Samples taken?
06	M					Y	Y	Y	Y	Y	Y	Y	Y		L	A			Red
	F					N	N	N	N	N	N	N	N		R	B			Grn
07	M					Y	Y	Y	Y	Y	Y	Y	Y		L	A			Red
	F					N	N	N	N	N	N	N	N		R	B			Grn
08	M					Y	Y	Y	Y	Y	Y	Y	Y		L	A			Red
	F					N	N	N	N	N	N	N	N		R	B			Grn
09	M					Y	Y	Y	Y	Y	Y	Y	Y		L	A			Red
	F					N	N	N	N	N	N	N	N		R	B			Grn
10	M					Y	Y	Y	Y	Y	Y	Y	Y		L	A			Red
	F					N	N	N	N	N	N	N	N		R	B			Grn

CATTLE BREED CODES:

WF= White Fulani **GU= Gudali**
RF= Red Fulani **EX= Exotic**
MX= Mixed Breed **OT= Other**
UN= Unknown

University of Edinburgh

Herd ID: ____/____

	ROB				HERDER									ROB					
ANIMAL ID	Gender	Breed	BCS	Dentition	Age(M/Y?)	Weight Loss?	Coughing	Diarrhoea	Not Eating	Breathing Difficulties	Worming (12 M)	Mastitis (FEMALE)	Abortion (FEMALE)	Comments	CST NECK SITE	PPD SITE	Day0 skin(mm)	Day3 skin(mm)	Samples taken?
11	M					Y	Y	Y	Y	Y	Y	Y	Y		L	A			Red
	F					N	N	N	N	N	N	N	N		R	B			Grn
12	M					Y	Y	Y	Y	Y	Y	Y	Y		L	A			Red
	F					N	N	N	N	N	N	N	N		R	B			Grn
13	M					Y	Y	Y	Y	Y	Y	Y	Y		L	A			Red
	F					N	N	N	N	N	N	N	N		R	B			Grn
14	M					Y	Y	Y	Y	Y	Y	Y	Y		L	A			Red
	F					N	N	N	N	N	N	N	N		R	B			Grn
15	M					Y	Y	Y	Y	Y	Y	Y	Y		L	A			Red
	F					N	N	N	N	N	N	N	N		R	B			Grn

CATTLE BREED CODES:

WF= White Fulani **GU= Gudali**
RF= Red Fulani **EX= Exotic**
MX= Mixed Breed **OT= Other**
UN= Unknown

Appendix G

Feedback poster


YOU CAN GET
TUBERCULOSIS FROM CATTLE:
PROTECT YOURSELF
COOK MEAT WELL
IF SICK
GET TESTED
BOIL MILK

Appendix H

Comparison of abattoir and pastoral cross-sectional study samples.

NORTH WEST REGION			
Division	Pastoral cattle registered at VC in 2012	Pastoralist cross-sectional study proportion of North West Region herds sampled (n=50)	Bamenda Abattoir study proportion of total cattle slaughtered (n=1129, 95% CI)
Boyo	9.5%	10.8%	19.7% (17.4-22.2%)
Bui	25.5%	25.7%	13.6% (11.6-15.7%)
Donga Mantung	25.5%	25.4%	11.0% (9.2-13.0%)
Menchum	9.9%	9.2%	30.8% (28.1-33.6%)
Mezam	15.7%	15.0%	8.0% (6.5-9.8%)
Momo	7.6%	7.5%	16.2% (14.1-18.5%)
Ngoketunjia	7.6%	6.5%	0.8% (0.4-1.6%)
VINA DIVISION			
Subdivision	Pastoral cattle registered at VC in 2012	Pastoralist cross-sectional study proportion of Vina Division herds sampled (n=50)	Ngaoundere Abattoir study proportion of total cattle slaughtered (n=930, 95% CI)
Belel	19.7%	20.1%	19.9% (17.2-22.9%)
Martap	35.3%	34.7%	1.8% (1.0-3.0%)
Mbe	2.9%	3.2%	0.0%
Ngan-Ha	9.6%	11.0%	7.8% (6.1-10.0%)
Ngaoundere I	1.8%	1.6%	0.5% (0.2-1.5%)
Ngaoundere II	1.5%	1.3%	21.5% (18.7-24.7%)
Ngaoundere III	4.6%	4.3%	6.9% (5.2-8.9%)
Nyambaka	24.6	23.9%	9.9% (7.9-12.2%)
Outside the Vina Division.			
Djohong, Mbere Division, Adamawa Region, Cameroon	NA	NA	0.1% (0.0-1.0%)
Touboro, Mayo Rey Division, North Region, Cameroon	NA	NA	17.4% (14.8-20.3%)
Tchollire, Mayo Rey Division, North Region, Cameroon	NA	NA	14.1% (11.8-16.9%)

Table H.1: **Proportions of pastoral cattle within the North West Region and Vina Division by VC cattle population data (2012 vaccination records).**
Proportion of herds sampled in the pastoral cross-sectional study and cattle slaughtered in the abattoir study. Subdivided by division in the North West Region and subdivision in the Vina Division.

Appendix I

Non-significant final multivariate logistic regression models for false positive and negative IFN-gamma assay results by abattoir.

BAMENDA						
(a) IFN-gamma assay POSITIVE subgroup (>=0.1, n=60)						
TB Lesion		lesionANPN∼1 + sex + dentition + breed				
		Variables	Levels	Odds ratio	95% CI	p value
+	-	sex	Male	1		
			Female	1.21	0.37-4.11	0.75
		dentition	<3 years	1		
			>=3 years	0.26	0.04-1.19	0.12
41	19	breed	Mixed breed	1		
			Fulani	4.04	0.85-22.71	0.08
NGAOUNDERE						
(a) IFN-gamma assay POSITIVE subgroup (>=0.1, n=48)						
TB Lesion		lesionANPN∼1 + sex * dentition + FgPathBin * breed				
		Variables	Levels	Odds ratio	95% CI	p value
+	-	sex	Male	1		
			Female	0.01	0.00-INF⋆	0.99
		dentition	<3 years	1		
			>=3 years	0.01	0.00-INF⋆	0.99
		breed	Mixed breed	1		
			Fulani	0.02	0.01-21.9	0.45
24	24	FgPathBin	Negative	1		
			Positive	0.15	0.01-1.00	0.10
		sex:dentition	Female:>=3 years	11.16	0.01-INF⋆	0.99
		breed:FgPathBin	Fulani:Positive	11.37	0.57-42.48	0.13
(b) TB Lesion NEGATIVE subgroup (n=687)						
IFN-gamma		bovigam.01∼1 + sex * dentition + FgPathBin + breed				
		Variables	Levels	Odds ratio	95% CI	p value
+	-	sex	Male	1		
			Female	0.38	0.04-8.20	0.43
		dentition	<3 years	1		
			>=3 years	0.01	0.00-INF⋆	0.99
		breed	Mixed breed	1		
			Fulani	1.19	0.42-2.99	0.72
25	662	FgPathBin	Negative	1		
			Positive	0.49	0.02-1.14	0.11
		sex:dentition	Female:>=3 years	1.21	0.00-INF⋆	0.99

Table I.1: **Final non-significant disagreement risk factor models for IFN-gamma assay false positives.**

1. Bamenda (a) Dependent variable TB lesion negative (lesionANPN) in IFN-γ positive sub group (n=60). 2. Ngaoundere (a) Dependent variable TB lesion negative (lesionANPN) in IFN-γ positive sub group (n=48). 3. Ngaoundere (b) Dependent variable IFN-γ positive (bovigam.01) in TB lesion negative sub group (n=687). Key: lesionANPN= TB lesion result (Positive or negative); bovigam.01= IFN-γ result (Positive or negative); sex= Sex of cattle (Male or Female); dentition= Age of cattle by DS (<3 years or $\geq$3 years); FgPathBin= *Fasciola* pathology score; breed= Breed of cattle (Mixed breed or Fulani breed); *= Interaction between variables; ⋆= Model does not converge due to inadequate numbers in an explanatory variable level. Note: Bamenda (b) Dependent variable IFN-γ positive (bovigam.01) in TB lesion negative sub group (n=1043) not shown as final model is the base model.

BAMENDA						
(c) IFN-gamma assay NEGATIVE subgroup (>=0.1, n=1023)						
TB Lesion		lesionANPN∼1 + sex + dentition + breed				
		Variables	Levels	Odds ratio	95% CI	p value
+	-	sex	Male	1		
			Female	2.27	0.94-5.67	0.07
		dentition	<3 years	1		
24	999		>=3 years	0.70	0.28-1.86	0.46
		breed	Mixed breed	1		
			Fulani	3.57	0.73-64.38	0.22
(d) TB Lesion POSITIVE subgroup (n=43)						
IFN-gamma		bovigam.01∼1 + sex + dentition + breed + OLS + cultureR				
		Variables	Levels	Odds ratio	95% CI	p value
+	-	sex	Male	1		
			Female	8.66	0.01-38.2	0.26
		dentition	<3 years	1		
			>=3 years	0.09	0.01-7.71	0.57
24	19	breed	Mixed breed	1		
			Fulani	2.94	0.00-INF⋆	0.99
		cultureR	Negative	1		
			M. bovis positive	0.01	0.00-INF⋆	0.99
			NTM positive	0.01	0.00-INF⋆	0.99
		OLS	-	1.22	0.25-6.18	0.79
NGAOUNDERE						
(d) TB Lesion POSITIVE subgroup (n= 77)						
IFN-gamma		bovigam.01∼1 + sex + dentition +FgPathBin + breed + OLS + cultureR				
		Variables	Levels	Odds ratio	95% CI	p value
+	-	sex	Male	1		
			Female	2.01	0.30-14.37	0.44
		dentition	<3 years	1		
			>=3 years	1.06	0.17-5.47	0.94
		FgPathBin	Negative	1		
			Positive	1.34	0.43-4.13	0.61
53	24	breed	Mixed breed	1		
			Fulani	0.16	0.04-4.89	0.22
		cultureR	Negative	1		
			M. bovis positive	0.82	0.19-3.23	0.78
			NTM positive	0.01	0.00-22.2	0.99
		OLS	-	0.71	0.35-1.36	0.31

Table I.2: **Final non-significant disagreement risk factor models for IFN-gamma assay false negatives.**

1. Bamenda (c) Dependent variable TB lesion positive (lesionANPN) in IFN-γ negative sub group (n=1023). 2. Bamenda (d) Dependent variable IFN-γ negative (bovigam.01) in TB lesion positive sub group (n=43). 3. Ngaoundere (d) Dependent variable IFN-γ negative (bovigam.01) in TB lesion positive sub group (n=77). Key: lesionANPN= TB lesion result (Positive or negative); bovigam.01= IFN-γ result (Positive or negative); sex= Sex of cattle (Male or Female); dentition= Age of cattle by DS (<3 years or $\geq$3 years); FgPathBin= *Fasciola* pathology score; breed= Breed of cattle (Mixed breed or Fulani breed); cultureR= Culture result (Negative, *M. bovis* positive or Non-tuberculous mycobacteria positive); OLS= Overall lesion score (Continuous variable); *= Interaction between variables; ⋆= Model does not converge due to inadequate numbers in an explanatory variable level.

Appendix J

Univariate logistic regression analysis for IFN-gamma positivity risk factors in pastoral and dairy cattle.

VARIABLE	Variable Description	Levels	Odds Ratio	95% CI	p value
ABREED	Breed	Mixed breed	1		
		Gudali*	0.46	0.3-0.69	<0.01
		Fulani	0.72	0.39-1.28	<0.02
ANIDEN	Dentition score (Re-grouped into age groups)	<3 years	1		
		>=3 years*	1.53	1.07-2.19	0.02
QUESWM	Wormed in the previous	No	1		
		Yes	0.84	0.58-1.2	0.31
ANISEX	Animal sex	Male	1		
		Female	0.76	0.53-1.1	0.13
NUMCTPc	Number of cattle presented (Re-grouped)	<25 cattle	1		
		25-50 cattle	1.84	0.95-3.93	<0.05
		>=50 cattle*	2.26	1.15-4.87	<0.05
SHEEPO	Sheep also kept at the homestead	No	1		
		Yes	1.41	0.99-2	0.05
GOATSO	Goats also kept at the homested	No	1		
		Yes	1.23	0.84-1.78	0.25
BIRDSO	Poultry also kept at the homested	No	1		
		Yes	0.94	0.64-1.38	0.72
CATTSO	Cats also kept at the homested	No	1		
		Yes	1.1	0.77-1.56	0.58
DOGGSO	Dogs also kept at the homested	No	1		
		Yes	1.15	0.79-1.65	0.44
HORSEO	Horses also kept at the homested	No	1		
		Yes	1.55	1.03-2.29	0.07
		Unknown	0.7	0.02-4.69	0.07
VACPRV	Vaccinated in the previous 12 months	Yes	1		
		No	1.37	0.15-6.12	0.68
TRYPRV	Trypanosome treatment used in the previous 12 months	Yes	1		
		No*	1.92	1.35-2.74	<0.01
AISBRD	Artificial Insemination (AI) used in the previous 12 months	No	1		
		Yes	0.98	0.42-2.01	0.96
GRZFLD	Grazed flooded pasture in the previous 12 months	No	1		
		Yes	0.77	0.54-1.10	0.32
		Unknown	0.85	0.16-2.87	0.32
GRZNHD	Graze with other herds in the previous 12 months	No	1		
		Yes*	0.19	0.12-0.32	<0.01
GRZBUF	Graze with buffalo in the previous 12 months	No	1		
		Yes	0.00	0.00-2.47	0.39
GRZANT	Graze with antelope in the previous 12 months	No	1		
		Yes*	0.45	0.31-0.64	<0.01
GRZHOG	Graze with warthog in the previous 12 months	No	1		
		Yes	0.82	0.53-1.25	0.34
HOUSEC	Cattle housed majority of the time	No	1		
		Yes	0.63	0.07-2.53	0.52
FENCEH	Cattle fenced in at night with other herds	Not fenced	1		
		Fenced with no other herds*	2.32	1.62-3.32	<0.01
		Fenced with 1-5 other herds	0.88	0.22-2.48	<0.01
DRKWTT	Cattle drink from water troughs	No	1		
		Yes	3.28	0.75-11.22	0.03
DRKSTR	Cattle drink from streams	No	1		
		Yes*	0.09	0.04-0.2	<0.01
DRKCON	Number of other herds in contact with at	0 herds	1		
		1-5 herds	0.84	0.58-1.21	0.36
		>5 herds	0.67	0.33-1.26	0.36
DRKSAM	Number of other herds using watering points	0 herds	1		
		1-5 herds*	0.32	0.20-0.53	<0.01
		>5 herds*	0.29	0.16-0.50	<0.01

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VARIABLE	Variable Description	Levels	Odds Ratio	95% CI	p value
TRACAT	Cattle undertake transhumance in the previous 12 months	No	1		
		Yes	0.96	0.63-1.44	0.84
SALCAT	Sold cattle in the previous 12 months	No	1		
		Yes	0.59	0.37-0.98	0.02
BUYCAT	Purchased cattle in the previous 12 months	No	1		
		Yes	0.92	0.64-1.30	0.62

Table J.1: **Univariate analysis for pastoral cattle risk factors for bovine tuberculosis using the IFN-gamma assay (n=1498).**

VARIABLE	Variable Description	Levels	Odds Ratio	95% CI	p value
ANIDEN	Dentition score (Re-grouped into age groups)	<3 years	1		
		>=3 years	0.7	0.09-8.59	0.65
QUESWM	Wormed in the previous 12 months	Y	1		
		N	0.87	0.02-11.83	0.68
ANISEX	Animal sex	Female	1		
		Male	0	0-107.7	0.54
SUBDIV	Cooperative group	Bamenda	1		
		Jakiri*	6.91	1.1-84.21	0.02
		Santa	1.61	0.24-18.52	0.02
NUMCTPc	Number of cattle presented (Re-grouped)	1-2	1		
		2-4	0.69	0.15-2.88	0.18
		5-6	3.94	0.38-55.35	0.18
SHEEPO	Sheep also kept at the homestead	No	1		
		Yes	2.33	0.54-9.51	0.18
GOATSO	Goats also kept at the homested	No	1		
		Yes	0.88	0.2-3.42	0.84
BIRDSO	Poultry also kept at the homested	No	1		
		Yes	1.52	0.4-6.55	0.50
CATTSO	Cats also kept at the homested	No	1		
		Yes	1.52	0.4-6.55	0.50
DOGGSO	Dogs also kept at the homested	No	1		
		Yes	0.69	0.19-2.57	0.53
HORSEO	Horses also kept at the homested	No	1		
		Yes	2.87	0.03-229.69	0.45
VACPRV	Vaccinated in the previous 12 months	Yes	1		
		No	0	0-107.70	0.54
WORDRG	Anthelmintic used in the previous 12 months	UNK	1		
		Albendazole	1.3	0.24-5.78	0.71
TRYPRV	Trypanosome treatment used in the previous 12 months	No	1		
		Unknown	1.43	0.12-11.22	0.70
NATBRD	Natural breeding used in the previous 12 months?	No	1		
		Yes	2.37	0.225-116.03	0.43
AISBRD	AI used in the previous 12 months	No	1		
		Yes	0.9	0.08-5.89	0.91
GRZOPN	Cattle grazed open pasture the	No	1		
		Yes	1.95	0.15-18.72	0.48
HOUSEC	Cattle housed majority of the time	No	1		
		Yes	0.16	0-3.47	0.11
DRKSAM	Number of other herds using watering points	0 herds	1		
		1-5 herds	1.95	0.15-18.72	0.48
SALCAT	Sold cattle in the previous 12 months	No	1		
		Yes	0.50	0.12-1.9	0.26
BUYCAT	Purchased cattle in the previous 12 months	No	1		
		Yes	0	0-1.84	0.09
BUYSAL	Places conducted sales or purchases of cattle in the past 12 months	Closed herd	1		
		Markets	0	0-5.34	0.27
		Others	0.34	0.05-1.59	0.27
		Unknown	1.26	0.09-12.85	0.27

Table J.2: **Univariate analysis for dairy cattle risk factors for bovine tuberculosis using the IFN-gamma assay (n=60).**

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